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No escalation of curative doses of reference drugs (chloroquine and primaquine) in the simian radical curative models has been recorded over the last 14 years. A shorter 3- dose regimen of WR 238605 for anti-relapse activity has been established which would be operationally more acceptable. In addition this compound has exhibited significant blood-schizontocidal, prophylactic and gametocytocidal activities which can be exploited. A new anti-relapse regimen comprising of WR 238605 plus halofantrine or mefloquine as companion blood schizontocides has been developed for management of chloroquine resistant P.vivax in the field. Cyproheptadine has shown antimalarial action against multidrug resistant P.yoelii nigeriensis. Cyproheptadine also exerts mefloquine resistance reversal action against P.knowlesi. This finding will be useful for treatment of mefloquine resistant cases. In vitro bioassay for evaluation of antimalarials using synchronized P.knowlesi and in vitro model for screening of tissue schizontocidal drugs using P.cynomolgi have been established. An in vitro test for methemoglobin toxicity using mastomys erythrocytes to evaluate toxicity of 8-aminoquinoline agents has been developed. Recombinant Hu IL-12 has shown promising prophylactic activity.

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Plasmodium cynomolgi, WR 238605, Halofantrine, Mefloquine, Chloroquine, Primaquine, Cyproheptadine, Anti-relapse, Drug combinations. Resistance reversal, P.Knowlesi in vitro bioassay, IL-12, [illegible] toxicity, [illegible]

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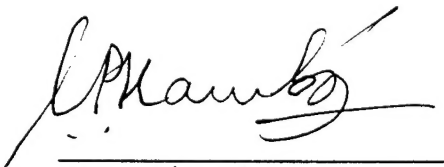

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CDRI-WRAIR COLLABORATIVE PROJECT
DAMD 17-93-J-3019

SUMMARY OF THE MAJOR ACHIEVEMENTS

1. CYCLIC TRANSMISSION OF THE MALARIA PARASITES

A. *P. cynomolgi* - Rhesus Monkey model

Plasmodium cynomolgi B is being maintained by cyclic transmission through *An. stephensi* mosquitoes and on an average the parasite undergoes a complete monkey-mosquito-monkey cycle in 40-45 days. The parasite has been maintained through 120 cyclic passages through *An. stephensi*.

B. *P. yoelii nigeriensis* (N-67) Swiss Mice model

The cyclic transmission of the rodent malaria parasite *P. yoelii nigeriensis* through *An. stephensi* mosquitoes has also been established. Golden hamster has been found to be suitable host for infective blood meal and gametocyte production for infection of mosquitoes, and maintenance of cyclic passage of this parasite.

2. REVALIDATION OF CHLOROQUINE AND PRIMAQUINE CURATIVE DOSES AGAINST *P. CYNOMOLGI* B

The curative blood schizontocidal dose of chloroquine (3 mg/kg base x 7 days), and causal prophylactic (1.78 mg/kg x 3 days) and anti-relapse curative doses of primaquine (1.00 mg/kg base x 7 days) have been revalidated and no escalation in curative doses established since 1982 has been observed. The protocols for blood schizontocidal test, causal prophylactic test, anti-relapse test, gametocytocidal/sporontocidal efficacy tests using *P. cynomolgi* B have been maintained operational during the tenure of the project.

3. BLOOD SCHIZONTOCIDAL CURATIVE DOSE OF CHLOROQUINE IN THE SHORTER THREE DOSE REGIMEN

Curative dose of chloroquine has also been determined using shorter three dose treatment schedule and the dose of 10.0 mg base/kg x 3 days by oral route has been found to be curative against *P. cynomolgi* B.

4. BLOOD SCHIZONTOCIDAL ACTIVITY OF ANTIMALARIALS

The curative blood schizontocidal dose of mefloquine, halofantrine and WR 242511 has been established against *P. cynomolgi* B in rhesus monkey model.

A. Mefloquine: The blood schizontocidal activity of mefloquine was evaluated against trophozoite induced *P. cynomolgi* B infection in rhesus monkeys and dose of 10 mg/kg x 7 days, administered orally was curative.

B. Halofantrine: Halofantrine at 10 mg/kg x 7 dose (oral) schedule was curative against blood induced *P. cynomolgi* infection.

C. WR 242511: The blood schizontocidal dose of WR 242511, a 5 methoxy 8-aminoquinoline against *P. cynomolgi* B was determined at 1.00 mg/kgx7 day.

5. ADDITIONAL ANTIMALARIAL DATA WITH COMPOUND WR 238605

Compound WR 238605 identified under CDRI-WRAIR collaborative programme has been selected by Walter Reed Army Institute of Research for Phase II clinical trials. This compound is a potential anti-relapse antimalarial which may eventually replace primquine. In the *P. cynomolgi* rhesus monkey model, this compound has shown 7-10 fold better therapeutic activity compared to primaquine and compound is safe for clinical trials.

A. Blood schizontocidal activity of WR 238605

Additional blood schizontocidal data has been obtained for compound WR 238605 against two simian malaria parasites namely *P. cynomolgi* B and *P. fragile*

and the new compound has shown 10 fold better blood schizontocidal activity than primaquine.

B. Radical curative activity of WR 238605 in the shorter three dose regimen

Compound WR 238605 was evaluated for anti-relapse activity using three dose treatment regimen and a dose of 1.00 mg/kg x 3 days was found to be curative.

C. Gametocytocidal activity of WR 238605

Compound WR 238605 has also shown significant gametocytocidal activity at 2 mg/kg single dose against *P. cynomolgi* B.

6. EVALUATION OF ALTERNATE REGIMENS FOR MANAGEMENT OF CHLOROQUINE RESISTANT *P. VIVAX* CASES

With the establishment of foci of chloroquine resistant *P. vivax* in several geographical regions, the management of this parasite is likely to pose problems in the coming years. Halofantrine and mefloquine are the alternate drugs which can possibly replace chloroquine as the blood schizontocidal agent. Compound WR 238605 is undergoing Phase II clinical trials as a replacement drug for primaquine, as the tissue schizontocidal agent. This new compound has shown improved efficacy and better half-life than primaquine in animal studies carried out earlier at CDRI. The rational for undertaking present study was to evaluate the compatibility of two alternate blood schizontocides, namely halofantrine/mefloquine with WR 238605 for management of chloroquine resistant *P. vivax* cases.

A. Combination studies with halofantrine and WR 238605

(i) Blood schizontocidal activity : Halofantrine shows curative blood schizontocidal activity against blood induced *P. cynomolgi* B infection at 10.0 mg/kg, while compound WR 238605 is also curative at 3.16 mg/kg dose in the standard 7 day blood schizontocidal test. Co-administration of WR 238605 at

0.316 mg/kg in combination with halofantrine at 3.16 mg/kg were found to be curative thereby indicating an additive or possibly synergistic effect of the combination on the blood stages of the parasite. The study shows that in combination the curative dose of halofantrine is reduced by one-third, and that of WR 238605 by one-tenth.

(ii) Anti-relapse activity: Combination studies carried out with the above antimalarials for anti-relapse activity against sporozoite induced *P. cynomolgi* B infection also showed that concurrent administration of 0.316 mg/kg WR 238605 (effective anti-relapse dose) along with 3.16 mg/kg halofantrine was curative as evidenced by absence of any relapse in the treated monkeys. The results show that halofantrine does not antagonise the anti-relapse activity of WR 238605 in the simian model and clinical trials with this combination could lead to a drug-regimen for the radical cure in chloroquine resistant *P. vivax* areas.

B. Combination studies with mefloquine and WR 238605

(i) **Blood schizontocidal activity**: Co-administration of 5.62 mg/kg mefloquine and 0.316 mg/kg WR 238605 was curative against blood stages of *P. cynomolgi* indicating additive response of the two components.

(ii) Anti-relapse activity: Concurrent administration of 0.316 mg/kg of WR 238605 and 5.62 mg/kg mefloquine also showed radical curative activity against sporozoite induced infections of *P. cynomolgi*, thus indicating the compatibility of the two agents for treatment of relapses. The study clearly establishes that the blood schizontocide chloroquine can be replaced by mefloquine in radical curative test. Clinical trials with mefloquine + WR 238605 combination in chloroquine resistant *P. vivax* areas are warranted.

7. ANTI-HISTAMINICS AS NEW CLASS OF BLOOD SCHIZONTOCIDES

Cyproheptadine has shown significant anti-malarial activity at 20 mg/kg dose against multi-resistant *P. yoelii nigeriensis*. Three other anti-histaminic

compounds tested did not show any activity. Cyproheptadine which is an anti-histaminic and 5-HT antagonist provides a new lead and its analogues can be exploited for potential anti-malarial activity and control of drug-resistant malaria.

8. DRUG RESISTANT STRAINS FOR RESISTANCE REVERSAL STUDIES

(a) **Simian malaria**

The following sub-lines of *P. knowlesi* W_1 have been initiated with a view to establish stable drug resistance.

(1) Chloroquine resistant strain: Efforts are continuing to establish chloroquine resistant strain of *P. knowlesi*, but so far resistance to chloroquine has not been established though the parasite was exposed to sub-curative doses of the drug *in vivo* for over one year period.

(2) Mefloquine resistant strain: has been developed and it can tolerate mefloquine up to 80 mg/kg x 3 doses. This strain will be useful for pre-clinical evaluation of mefloquine resistance reversal agents such as penfluoridol and other potential reversal agents. The mefloquine resistant *P. knowlesi* has been cryopreserved.

b) Rodent malaria

(i) The following drug resistant lines of rodent malaria parasite *P. berghei* have been cryopreserved.

1. Chloroquine resistant strain (resistant up to 128 mg/kgx4)
2. Mefloquine resistant strain (resistant up to 128 mg/kgx4)
3. Quinine resistant strain (resistant up to 400 mg/kgx4)

(ii) A multiple resistant strain of *P. yoelii nigeriensis* resistant to chloroquine (128 mg/kgx4), mefloquine (128 mg/kg x 4) and quinine (400 mg/kgx 4) has been cryopreserved. This strain has been used for resistance reversal studies as it produces 100% lethal infection.

(iii) Additional drug resistant strains of mosquito transmissible *P. yoelii nigeriensis* (N-67) have been selected in the Swiss mice model.

(a) Chloroquine resistant strain 128 mg/kg

(b) Mefloquine resistant strain 128 mg/kg

(c) Halofantrine resistant strain 128 mg/kg

(d) Pyrimethamine resistant strain 48 mg/kg

The stability of the above resistant strains after transmission through the vector (*A. stephensi*) has been established. The strains would be useful for resistance reversal studies, and would serve as primary *in vivo* screens for resistance reversal activity.

9. STUDIES ON REVERSAL OF DRUG RESISTANCE

Several resistance reversal agents have been published in literature but in most of the studies the reversal effect was observed against *in vitro* cultures of chloroquine resistant *P. falciparum*. Studies have ben carried out to validate the resistance reversal effect in drug-resistant rodent malaria model (*P. yoelii nigeriensis*).

A. Resistance reversal studies with multi-drug resistant *P. yoelii nigeriensis*

(i) WR 238605 + chloroquine combination

The marginal extension of MST by 2 days was observed when WR 238605 (0.5 mg/kg) was given together with chloroquine (4.0 or 8.0 mg/kg), as compared to MST of control/chloroquine group suggesting some additive effect of WR 238605 when combined with chloroquine.

(ii) WR 238605 + Mefloquine combination

WR 238605 (at 0.5 mg/kg) did not potentiate the effect of mefloquine against mefloquine resistant strain. However, the combination exerts additive antimalarial effect as shown in the therapeutic (post-treatment) regimen.

(iii) **Verapamil**

Verapamil at higher doses provided a definitive extension of MST when the drug was given together with chloroquine. Studies show a limited chloroquine resistance reversal effect of verapamil in day 2-6 treatment schedule.

iv) **Nifedipine**

Nifedipine at 10-15 mg/kg given with chloroquine 8 mg/kg resulted in extension of mean survival time to 24.7-24.8 days compared to 21.14 days of chloroquine control group, indicating some chloroquine resistance reversal effect of Nifedipine against multiple resistant rodent model used in this study.

v) **Quinidine**

Quinidine in combination with chloroquine exerts a possible additive action and no resistance reversal action was recorded.

B. Resistance reversal studies with mosquito transmissible *P. yoelii nigeriensis* (N-67) Swiss mice

P. yoelii nigeriensis (N-67 strains) resistant to chloroquine and mefloquine were selected after interrupted sub-curative therapy and resistance was found to be stable after transmission through vector.

i) **Verapamil**

Verapamil in combination with chloroquine, mefloquine or halofantrine shows low level of resistance reversal activity as shown by suppression of parasitaemia on day 4.

ii) **Amitryptiline**

Combination of amitryptiline with chloroquine, mefloquine or halofantrine showed only transient suppression of parasitaemia on day 4 and 7 in the combination treated groups.

iii) **Cyproheptadine**

Cyproheptadine has shown promising resistance reversal action against

chloroquine and halofantrine resistant strains as the combination treated animals were completely protected. Cyproheptadine has also significant activity in combination with mefloquine against mefloquine resistant strain.

C. Resistance reversal studies in simian malarial model (*P. knowlesi* rhesus monkey)

Cyproheptadine has been found to show resistance reversal activity against mefloquine resistant *P. knowlesi* in rhesus monkeys. Combination of 20 mg/.kg mefloquine x 3 days plus 10 mg/kg cyproheptadine x 5 days protected the treated monkeys while the two components individually are not curative. This is a promising lead where cyproheptadine has shown resistance reversal action against mefloquine resistant parasite. Mefloquine alone upto 80 mg/kg x 3 days does not cure *P. knowlesi*.

10. *IN VITRO* CULTIVATION AND BIOASSAY FOR ANTIMALARIALS

A. *In vitro* cultivation of *P. falciparum*

In vitro anti-malarial screening protocol against *P. falciparum* is being standardized using Giemsa staining of culture smears to monitor the parasiticidal dose end-point.

B. *In vitro* cultivation of simian parasite *P. knowlesi*

Over the years culture adapted parasites have found major application in evaluation of novel chemotherapeutic agents and drug combinations. One of the major limitations in wider use of *P. falciparum* cultures in the developing countries has been the poor availability of quality human serum which is indispensable for maintaining the continuous cultures. Hence studies were undertaken to standardize *in vitro* model using *P. knowlesi* parasites for evaluation of potential chemotherapeutic agents. The various factors which influence the parasite maturation have been optimized and base line data with reference anti-malarials has been obtained.

C. *In vitro* bioassay for anti-malarials using simian parasite *P. knowlesi*

Short term *in vitro* culture of *P. knowlesi* has been standardized using the candle jar technique and base-line data on ^{3}H-hypoxanthine incorporation at varying concentration of parasitaemia and haematocrit has been obtained using 24 hour incubation period. The application of this model for *in vitro* assay of potential anti-malarials using Giemsa stained blood smears has also been standardised for comparison.

D. Development of *in vitro* anti-malarial assay system using parasite LDH

An *in vitro* system for anti-malarial assay based on possible inhibition of the parasite LDH activity is being standardized using NAD and APAD as cofactors for the LDH biochemical assay. The LDH assay with APAD as substrate has been found to be very sensitive and this could be exploited as an *in vitro* screen for potential blood schizontocides.

E. *In vitro* tissue schizontocidal screening model

For *in vitro* screening of prospective tissue schizontocides, technology to obtain primary monkey hepatocyte cultures and development of exo-erythrocytic stages following inoculation of *P. cynomolgi* B sporozoites has been established. Primaquine at 0.1 μg/ml has been found to inhibit development of primary e-e schizonts.

11. *IN VITRO* METHEMOGLOBINS TOXICITY ASSAY

A simple and rapid *in vitro* assay using mastomys erythrocytes has been established to compare the relative toxicity of 8-aminoquinoline antimalarials.

12. MALARIA PROPHYLAXIS WITH RECOMBINANT IL-12 AGAINST *P. CYNOMOLGI* B SPOROZOITE CHALLENGE (COLLABORATION WITH NAVAL MEDICAL RESEARCH INSTITUTE, BETHESDA, DEPARTMENT OF U.S. NAVY)

A single dose of 10 μg/kg of recombinant human IL-12 (rHuIL-12) administered 2 days before challenge with *Plasmodium cynomolgi* sporozoites protected 7 out of 7 rhesus monkeys against malaria. Protection was associated with increase in circulating IFN-r and IFN-r, IL-6, IL-10, IL-12, IL-15 and TNF-α mRNA. It is believed that IL-12 protects monkeys through IFN and nitric oxide dependent elimination of infected hepatocytes. This first report of IL-12 induced protection of primates against an infectious agent supports assessment of rHuIL-12 for immunoprophylaxis against human malaria.

PROGRESS OF WORK (FEBRUARY 1993-FEBRUARY, 1997)

1. CYCLIC TRANSMISSION OF MALARIA PARASITES

A. Cyclic passage of *P. cynomolgi* B

The transmission of simian parasite *P. cynomolgi* through the vector has been maintained and the parasite has undergone 120 sequential passage since the initiation of the WRAIR-CDRI collaborative project in 1982. The details of serial passages (87-120) maintained during the period of report are summarized in Table 1.

The parasite has given high infectivity in *Anopheles stephensi* (colony bred). The insectary is maintaining 2000-3000 pupae/day under standard insectary conditions. Adequate numbers of sporozoites can be produced for accomplishing the tasks involved in prophylactic and radical curative tests. Prepatent period in rhesus monkeys after sporozoite inoculation ($0.26x10^6$ to $1.54x10^6$) has been recorded to range between 7-9 days.

B. Cyclic passage of *P. yoelii nigeriensis* (N-67)

A gametocyte producing strain of *P. yoelii nigeriensis* was obtained from Malaria Research Centre, Delhi and the optimum conditions for the transmission of this parasite through *A. stephensi* mosquitoes have been established. Hamster has been found to be a suitable host for obtaining gametocytes for infectivity studies. This model will be useful for prophylactic studies involving drug resistant parasites.

2. REVALIDATION OF CHLOROQUINE AND PRIMAQUINE CURATIVE DOSES

A. Chloroquine blood schizontocidal dose

The curative dose of chloroquine against blood induced *P. cynomolgi* B infection was established as 5 mg base/kg x 7 days (oral). The treated monkeys

are observed for 60 days after the end of treatment and absence of any recrudescence during this period indicates curative activity. The dose of chloroquine was revalidated several times during the last 4 years and no escalation in curative dose has been recorded.

Three day regimen: Three day regimen of chloroquine was evaluated against blood induced infection of *P. cynomolgi* B using 5.0, 7.5 and 10.0 mg/kg doses of chloroquine (base) administered orally for three consecutive days. Results in TAble 2 show that 10.0 mg/kg was the curative dose, 7.5 mg/kg was curative in one out of two monkeys and 5.0 mg/kg failed in both the monkeys. The monkeys which showed recrudescence in the above study were again treated at 7.5 mg/kg and 10.0 mg/kg and both these doses were curative.

B. Primaquine prophylactic and radical curative dose

The causal prophylactic dose of primaquine (1.78 mg base/kgx3 days) was revalidated against sporozoite induced *P. cynomolgi* B infection in 2 monkeys and both the monkeys were cured (Table 3).

Radical curative dose of primaquine (1 mg/kg base x 7 days) was revalidated in 2 monkeys each during 86th and 90th serial passages and dose was found to be curative. The lower dose 0.316 mg/kgx7 days used during 86th serial passage was not curative as expected and monkeys relapsed on day 29 and 37 (Table 4 and 5).

The curative blood schizontocidal dose of chloroquine and causal prophylactic and radical curative doses of primaquine, have shown no escalation during the last 15 years.

3. BLOOD SCHIZONTOCIDAL ACTIVITY OF MEFLOQUINE, HALOFANTRINE AND WR 242511

A. Blood schizontocidal activity of Mefloquine

The blood schizontocidal activity of mefloquine was evaluated in 2 monkeys each at 3.16 mg/kg, 10 mg/kg and 31.6 mg/kgx7 days. The lower dose of 3.16

mg/kg failed to clear the parasitaemia in both the monkeys while parasite clearance was recorded in 72-96 hours in monkeys treated at higher doses. There was no recrudescence in any of the monkeys treated at 10.0 and 31.6 mg/kg till 60 days (Table 6). The dose of 10 mg/kg was revalidated in 2 naive monkeys and both were protected (Table 7).

B. Blood Schizontocidal activity of Halofantrine

The blood schizontocidal activity of Halofantrine was evaluated in 2 monkeys each at 3.16 mg/kg, 10.00 and 31.6 mg/kgx7 days. The parasite clearance in all the monkeys was observed between 48-72 hours. The lowest dose of 3.16 mg/kg was not curative as indicated in Table 8. Monkeys at the higher dose i.e. 10.00 and 31.6 mg/kgx7 days were cured and did not show any recrudescence. Activity at 10 mg/kgx7 days was revalidated in 2 monkeys (Table 9), and it was found to be curative. Further tests were carried out at 5.6 mg/kgx7 days dose schedule in four monkeys, and the compound was curative at this dose in three out of four monkeys, while the fourth showed recrudescence. Test carried out at 10 mg/kgx7 days, was curative in both the monkeys (Table 10).

C. Blood schizontocidal activity of WR 242511

The blood schizontocidal activity of WR 242511 was evaluated in 2 monkeys each at 0.316 mg/kg, 1.00 mg/kg and 3.16 mg/kgx7 days. The lowest dose of 0.316 mg/kg was not curative as indicated in Table 11. Monkeys at the doses of 1.00 and 3.16 mg/kgx7 days were cured and have not shown recrudescence during 60 days observation period. Revalidation of 1 mg/kgx7 days dose showed that the dose was curative in two monkeys (Table 12).

In view of the sporadic emergence of chloroquine resistant *P. vivax* parasites, the treatment of resistant cases would need the shifting of chloroquine therapy to an alternate blood schizontocide for use as companion drug with the radical curative agent like primaquine or the new compound WR 238605 which is under clinical phase II trials at Walter Reed. Amongst the alternate blood

schizontocides which can replace chloroquine include mefloquine and halofantrine. With a view to establish their efficacy, the data generated with these compounds clearly show that mefloquine and halofantrine are curative as blood schizontocides at 10 mg/kgx7 days.

4. ADDITIONAL ANTIMALARIAL DATA WITH COMPOUND WR 238605

Preclinical evaluations carried out earlier at CDRI with compound WR 238605 had demonstrated this new compound to be 7-10 fold more active as causal prophylactic or radical curative drug. The radical curative dose was established at 0.316 mg/kgx7 days in the *P. cynomolgi* rhesus monkey model and the data was pivotal to the design of Phase I and Phase II clinical investigations now being carried out in Thailand by WRAIR. Additional studies have been carried out with this compound to establish its additional spectrum of activity as blood schizontocidal agent and gametocytocidal agent. Besides, the radical curative dose of this compound using shorter 3-dose regimen has also been established.

A. Blood schizontocidal activity against *P. cynomolgi* B and *P. fragile*

The blood schizontocidal activity of compound WR 238605 has been evaluated against two simian parasites *P. cynomolgi* B and *P. fragile*. Results in Table 13 show that against *P. cynomolgi* B infection, 10 out of 12 monkeys were protected at 1 mg/kg dosex7 days. All the 6 monkeys treated at 3.16 mg/kgx7 were also cured. In comparison primaquine was not curative in any of the 4 monkeys at 3.16 mg/kgx7 and in 3 out of 4 monkeys at 10 mg/kgx7 days. Likewise, against *P. fragile* infection, compound WR 238605 cured 10 out of 11 monkeys at 1 mg/kg and all the 4 monkeys at 3.16 mg/kgx7 days. Primaquine protected 1 out of 3 monkeys at 3.16 mg/kgx7 and 2 out of 3 monkeys at 10 mg/kg dose x 7 days. The study concludes that compared to the primaquine, compound WR 238605 has shown 10 fold higher blood schizontocidal activity against *P. cynomolgi* B and *P. fragile* infections in rhesus monkey models.

B. Gametocytocidal activity of WR 238605 against *P. cynomolgi* B

For the gametocytocidal test, batches of 3-4 day old *An. stephensi* were allowed to feed on *P. cynomolgi* infected rhesus monkeys at appropriate gametocytaemia level. Our earlier studies have shown that the sequential feeding of healthy mosquitoes on 3-4 consecutive days during the declining phase of the secondary asexual peak parasitaemia gave consistently good infectivity. One hr after the control (pre-treatment) feeding, compound WR 238605 was administered to the monkeys at 1.0, 2.0 and 4.0 mg(base)/kg in a single dose by oral route. Post-treatment feeding of batches of healthy mosquitoes was done at different times (6-8 hr). Mosquitoes were maintained at 26±1°C under optimal insectary conditions. The infectivity rate and the oocyst counts were recorded on day 8. Mosquitoes were further maintained in the insectary upto day 15 to determine the formation of sporozoites in the experimental batches.

RESULTS

The gametocytocidal activity of WR 238605 was evaluated in 7 rhesus monkeys and the pre-treatment mosquito infectivity results for these monkeys show that the oocyst number of different batches ranged from 17.13±10.01 to 35.32±13.34 and the percent infectivity varied between 64.10 to 86.49% (Table 14). Sequential mosquito feedings on three monkeys treated at 1.00 mg/kg dose showed that there was no significant reduction in oocyst number and the percent infectivity in +6 hr mosquito batches for all the three monkeys and in +24 hr post-treatment batches for two out of three monkeys when compared to the corresponding control feeding at -1 hr. Salivary gland dissections of the mosquitoes from these batches on day 15 showed the presence of sporozoites, thus indicating that oocysts completed normal sporogonic development. No oocysts were observed over the midguts from mosquitoes fed at +48 hr after drug administration nor were any sporozoites seen in their salivary glands.

Identical results were obtained in the efficacy tests at 2.0 mg/kg in 3/3 monkeys and at 4.0 mg/kg in one monkey. The mosquito batches fed at +6 hr post-treatment showed no significant alteration in the oocyst numbers, and these oocysts were able to complete the sporogonic cycle as indicated by the presence of sporozoites in salivary glands on day 15-16. The mosquito batches fed on these monkeys at +24 hr did not develop any oocysts nor were any sporozoites demonstable in their salivary glands.

C. Shorter three dose regimen for radical curative activity

Compound WR 238605 had been earlier found to show anti-relapse activity at 0.316 mg/kg dose in the seven day regimen. Studies were carried out to determine the curative dose of the compound in "Three dose Regimen". Two monkeys each were treated at 0.50 mg/kg, 1.0 mg/kg and 2.00 mg/kg x 3 days. Monkeys treated at 0.50 mg/kg relapsed on days 25 and 43 while monkeys treated at higher doses were protected. In the second experiment, 3 monkeys treated with 0.75 mg/kg x 3 days were also protected (Table 15).

5. COMBINATION STUDIES WITH COMPOUND WR 238605 AND HALOFANTRINE

In view of the sporadic emergence of chloroquine resistant *P. vivax*, the treatment of resistant cases would need the shifting of chloroquine therapy to an alternate blood schizontocide for use as companion drug with the radical curative agent like primaquine or the new compound WR 238605 which is under clinical phase I trials at Walter Reed. Amongst the alternate blood schizontocides which can replace chloroquine include mefloquine and halofantrine. With a view to etablish their efficacy, the data generated with these compounds clearly showed that mefloquine and halofantrine are individually curative as blood schizontocides at 10 mg/kgx7 schedule against blood induced *P. cynomolgi* infection in rhesus monkeys. The reference blood schizontocidal drug chloroquine is curative in this model at 3.00 mg/kg x 7 day.

Further studies have been carried out using halofantrine in combination with the anti-relapse antimalarial WR 238605 in both the blood schizontocidal test and the radical curative test with a view to see whether the combination has additive/antagonistic effect.

A. Blood schizontocidal activity of WR 238605 and halofantrine combination

Studies with these compounds when used individually had shown that compound WR 238605 is curative at 3.16 mg/kgx7 days and halofantrine is curative at 10.0 mg/kgx7 days. Concurrent administration of WR 238605 at 0.316 mg/kg and halofantrine at 3.16 mg/kgx7 days protected two out of two monkeys, while WR 238605 at 0.316 mg/kg in combination with halofantrine at 1.00 mg/kg was not curative in any of the two monkeys (Table 16). Results indicate that the combination shows additive/synergistic effect as the curative doses of the components in the combination have been lowered by 10 and 3 fold respectively (Table 17).

B. Radical curative activity of WR 238605 and halofantrine combination

To evaluate the anti-relapse efficacy of WR 238605 in combination with halofantrine as the companion blood schizontocide, two monkeys each were treated with a combination of WR 238605 and halofantrine at 0.316 mg/kg + 3.16 mg/kg, 0.316 mg/kg+5.62 mg/kg and 0.316 mg/kg+10.0 mg/kgx7 days respectively. Follow up of these monkeys till 100 days showed that none of the monkeys relapsed thus indicating the curative efficacy of the doses (Table 4). In the second experiment, compound WR 238605 at 0.316 mg/kg dose was evaluated in combination with halofantrine at 1.78 mg/kg, 3.16 mg/kg and 5.62 mg/kg doses in two monkeys each. While one monkey at 0.316 mg WR 238605 + 1.78 mg/kg halofantrine relapsed on day 26, the other five monkeys were cured (Table 19). One monkey treated with compound WR 238605 alone at 0.316 mg/kg relapsed on

day 26. Additional two monkeys treated with Wr 238605 at 0.1 mg/kg and halofantrine at 10 mg/kg also relapsed on days 13 and 15. The efficacy of combination of 0.316 mg/kg 238605 + 3.16 mg/kg halofantrine was revalidated in 2 monkeys in the third experiment (Table 20) and the dose was again found to be curative. The summarized data of combination studies is presented in Table 20 and results show that halofantrine does not antagonize with the anti-relapse activity of compound WR 238605.

6. COMBINATION STUDIES WITH COMPOUND WR 238605 AND MEFLOQUINE

A. Blood schizontocidal activity of mefloquine and WR 238605 combination

The blood schizontocidal efficacy of mefloquine with a combination of WR 238605 was calculated at two dose levels. Two monkeys treated with 3.16 mg/kg mefloquine plus 0.316 mg/kg WR 238605 recrudesced on day 25 and 26, while two monkeys treated with combination of 5.62 mg/kg mefloquine and 0.316 mg/kg 238605 were protected. Another two monkeys treated with mefloquine alone at 5.62 mg/kg dose also recrudesced on days 12 and 17. The results suggest additive response of the two antimalarials (Table 21).

B. Radical curative activity of WR 238605 using mefloquine as the companion blood schizontocide

For the radical curative test four monkeys were administered 0.316 mg/kg 238605. Mefloquine at two dose levels (viz. 5.62 mg/kg and 10.0 mg/kg) was administered as the companion blood schizontocide using two monkeys for each dose. The results showed that WR 238605 (0.316 mg/kg) plus 10 mg/kg mefloquine, as well as WR 238605 (0.316 mg/kg) plus 5.62 mg/kg mefloquine, were curative as antirelapse regimen (Table 21). Mefloquine alone at 10 mg/kg dose showed relapse on days 11 and 12, as expected. The results show that mefloquine does not antagonise the antirelapse efficacy of compound WR 238605.

7. ANTIHISTAMINICS AS A NEW CLASS OF ANTIMALARIALS

Four anti-histaminic drugs namely Terfenadine, Mebhydrolin, CDRI 73/602 (anti-histaminic compound under phase II clinical trials), and cyproheptadine, were evaluated for their antimalarial potential against *P. yoelii nigeriensis* resistant to chloroquine, mefloquine and quinine. The results presented in Table 23 show that the cyproheptadine possesses exceptionally high anti-malarial activity at 20 mg/kg dose, giving 50% survival of the mice beyond 21 days. The mean survival time of the control group was 7.25 days, while the 20 mg/kg cyproheptadine extended the MST to more than 16 days. This is a new lead and it is proposed to get some new analogues of cyproheptadine as well as other antihistaminics and 5-HT antagonists tested for their antimalarial activity. The other three antihistaminics tested did not show any antimalarial action (Table 23).

8. DRUG RESISTANT SIMIAN MALARIA STRAINS

A. Selection of chloroquine resistant strain of *P. knowlesi*

i) Selection by relapse technique

Attempts were made to select a chloroquine resistant strain of *P. knowlesi* W_1 by sequential treating the infected monkeys at high parasitaemia level and the surviving parasites were inoculated into naive monkeys 24-48 hr after drug exposure. In the first passage, a monkey was treated at 25 mg total dose. The drug dose was gradually increased in 12 successive passages over a period a 174 days and a dose of 150 mg (total dose) was administered in the 12th passage. Several isolates were cryopreserved in different passages to check the chloroquine sensitivity at intervals. The parent strain (W_1) of *P. knowlesi* has been found to be curative at 7.5 mg base/kg chloroquine x 3 days. The chloroquine sensitivity of isolates cryopreserved during 11th passage was determined at 10.0, 15.0 and 20.0 mg/kgx3 days. The results showed that the parasite was resistant to a dose of 10 mg/kgx3 as treated monkey recrudesced 11 days after end of treatment. The level

of resistance was revalidated in two monkeys and stable resistant line could not be established.

ii) Selection by interrupted subcurative therapy

Attempts have also been made to select a chloroquine resistant strain of *P. knowlesi* by administering subcurative doses of chloroquine at interrupted intervals so as to allow constant drug exposure to the parasite. The first rhesus (Rh-1) was exposed to 5 doses of chloroquine ranging between 0.5-0.3 mg/kg during 8 days after which parasites were transferred to the naive monkey (Rh-II). Rhesus RH-II was exposed to 25 doses of chloroquine ranging between 0.2-0.3 mg/kg. The parasite has been subsequently passaged in four naive monkeys Rh III, Rh IV, Rh V and Rh VI as indicated in Figs. 1-6 and the subcurative chloroquine therapy was continued (Table 24). The strain was maintained under constant drug pressure for nearly 14 months (Figs. 1-6). The periodic sensitivity tests performed periodically indicated no escalation of chloroquine curative dose of 7.5 mg/kg chloroquine base x 3 days.

B. Selection of Mefloquine resistant *P. knowlesi* in rhesus Monkey

Three rhesus monkeys No. 1, 2 and 3 were infected with *Plasmodium knowlesi* (W_1 strain) by inoculating $1x10^6$ parasitized RBC intravenously. The thick and thin blood smears stained with Giemsa stain were observed for recording parasitaemia. The three monkeys were treated with different doses of mefloquine (80, 40 and 20 mg/kgx3 doses) by oral route.

Monkey No. 1:

On day 3 of infection when the parasitaemia was approximately 0.7% a dose of 80 mg/kg mefloquine hyrochloride was administered for 3 consecutive days. The monkey was parasite negative after the second dose. The parasitaemia showed recrudescence 55 days after the third dose of mefloquine. The parasitaemia rose to 2.5 and 8.0% on day 58 and 60 respectively (Fig. 7). On day

60 the monkey was treated with 7.5 mg/kg chloroquine (base) orally for 3 successive days with a view to determine the sensitivity of the parasite to chloroquine. The monkey remained negative after chloroquine treatment till follow up of 40 days. The parent line resistant to 80 mg/kg dose of mefloquine has been cryopreserved for resistance reversal study.

Monkey No. 2:

When the initial parasitaemia was 0.5%; the monkey was treated orally with 40 mg/kg, mefloquine hydrochloride for 3 consecutive days. The parasitaemia became -ve after the second dose, but there was recrudescence on day 11 after the last dose of mefloquine. This monkey was again treated with 40 mg/kg mefloquine orally for 3 consecutive days and was cured (Fig. 8).

Monkey No. 3:

The 3rd monkey with 0.3% parasitaemia, was treated with 20 mg/kg mefloquine hydrochloride orally for 3 consecutive days. The monkey was negative after the second dose. There was recrudescence on day 9 of the last dose of mefloquine. The monkey was again treated with 20 mg/kg mefloquine orally for 3 consecutive days. The monkey showed absence of parasitaemia after the second dose. On day 8 after the last dose, the monkey showed recrudescence. 20 mg/kg mefloquine was again administered orally for 3 consecutive days. The monkey was cured after the third dose of mefloquine but there was recrudescence and the parasitaemia reached 1.4% on day 14 after the last dose of mefloquine. The parasitized RBC were preserved in liquid nitrogen. The monkey was cured with 7.5 mg/kg chloroquine base x 3 day orally (Fig. 9).

9. DRUG RESISTANT RODENT MALARIA STRAINS

(i) The following drug resistant lines of rodent malaria parasite *P. berghei* have been cryopreserved.

1. Chloroquine resistant strain (resistant upto 128 mg/kgx4 doses)
2. Mefloquine resistant strain (resistant upto 128 mg/kgx4 doses).

3. Quinine resistant strain (resistant upto 400 mg/kgx4 doses).

(ii) A multiple resistant strain of *P. yoelii nigeriensis* resistant to chloroquine (128 mg/kgx4), mefloquine (128 mg/kgx4) and quinine (400 mg/kgx4) has been cryopreserved.

10. STUDIES ON REVERSAL OF DRUG RESISTANCE

A large number of reports have appeared in literature during the last decade in which the chloroquine resistance of the cultured drug resistant isolates of *P. falciparum* had been claimed to be reversible *in vitro* by certain agents/compounds designated as reversal agents/resistance modulators/MRD modifiers. In presence of resistance reversal agents, a much lower dose of chloroquine is required to kill the resistant *P. falciparum* in culture. So far, very few drug resistance reversal studies have been carried out in the *in vivo* malaria models. But the published data do not prove conclusively that available resistance reversal agents would be potentially safe clinically and effective. Efforts were, therefore, continued to establish chloroquine/mefloquine resistant simian malaria secondary screening models to evaluate these claims and also to complete preclinical studies on a few selected reversal agents, which could be identified as potential candidate compounds for clinical trials.

A. Drug resistance reversal studies against multi-resistant *P. yoelii nigeriensis*

This strain is resistant to chloroquine (128 mg/kg x 4), mefloquine (128 mg/kg x 4) and also quinine (400 mg/kg x 4) and it is 100% lethal for Swiss mice.

VERAPAMIL

Verapamil which is a calcium channel blocker has been evaluated for chloroquine resistant reversal activity against multiresistant *P. yoelii nigeriensis*. Two drug administration schedules from day 0-3 and day 3-6 post-infection were used.

(i) Day 0-3 treatment

Chloroquine alone was given at 8 mg/kg dose, verapamil at 25 mg/kg. Besides a combination of verapamil 10 and 25 mg/kg with 8 mg/kg dose of chloroquine was tested (Table 25). Mean survival time (MST) of verapamil and chloroquine combination was slightly extended (12.25-12.63 days) in comparison to MST of 10.75 days observed in chloroquine control group. Extension of MST was observed only at higher doses of verapamil (10 and 25 mg/kg) and no extension of MST was observed with lower dose of verapamil (0.5 and 1.0 mg/kg).

(ii) Day 3-6 treatment

In this second group, the drug administration schedule was from day 3-6 post-infection. Chloroquine treated group of mice showed MST 21.14 days whereas different doses of verapamil with 8 mg/kg chloroquine showed mean survival time ranging from 15.17, 23.67, 24.60 to 25.57 days and the increase in MST was directly related to the increasing dose of verapamil from 5-25 mg/kg (Table 26). The study shows a limited reversal effect of verapamil when given with chloroquine. It may be pointed out that the number of animals surviving with combination of verapamil and chloroquine has not been consistent in different experiments.

NIFEDIPINE

This drug was also tested in combination with chloroquine against multi-drug resistant *P. yoelii nigeriensis* using 3-7 day post-infection treatment schedule. Before drug treatment the parasitaemia was 0.5%. In groups given nifedipine + 8 mg/kg chloroquine, the maximum survival time was 24.7 and 24.8 days in comparison to chloroquine alone which gave 21.14 days (Table 27). In conclusion the nifedipine has provided marginal extension of MST, specially at the high dose.

EVALUATION OF WR 238605 FOR CHLOROQUINE RESISTANCE REVERSAL ACTION

For resistance reversal studies with WR 238605, chloroquine resistant strain of *P. yoelii nigeriensis* was used. Chloroquine treatment (4.0 and 8.0 mg/kg x 4 days) resulted in MST of 12.8±4.2 and 17.8±9.4 days respectively, while chloroquine at 4.0 and 8.0 mg/kg when given together with 0.5 mg/kg of WR 238605, resulted in only slight extension of MST from 12.8±4.2 to 14.4±5.9 at 4.0 mg chloroquine dose, and from 17.8±9.4 days to 19.4±9.0 days at 8.0 mg chloroquine dose. Administration of WR 238605 (0.5 mg/kg) with chloroquine (4.0 or 8.0 mg/kg) extended the MST by nearly 2 days at both the dose levels of chloroquine used in the study (Table 28).

The marginal extension of MST when WR 238605 is administered with chloroquine suggests some additive effect of the drug combination specially when both the drugs are blood schizontocides.

EVALUATION OF WR 238605 FOR MEFLOQUINE RESISTANCE REVERSAL ACTION

Day 0-3 treatments

Resistance reversal effect of WR 238605 (0.5 mg/kg dose) alone and in combination with various doses of mefloquine (1.0, 2.0, 4.0 and 8.0 mg/kg x 4 days) was evaluated using multi-resistant *P. yoelii nigeriensis*. This rodent parasite is resistant to mefloquine at 128 mg/kg x 4 days schedule. WR 238605 (0.5 mg/kg) alone did not extend the mean survival time of the mice which was 6.2 days compared to 5.8 days in control group (Table 29). Mefloquine alone (1.0-8.0 mg/kg doses) produced gradual increase of MST from 6.6 days to 15.0 days corresponding to increasing dose levels of mefloquine. When mefloquine doses (1.0, 2.0, 4.0 and 8.0 mg/kg) were given together with fixed dose of WR 238605 (0.5 mg/kg) ther was no increase in MST which varied from 6.6, 10.0, 11.0 to

13.2 days respectively corresponding with the increasing dose level of mefloquine. The study shows no significant resistance reversal effect of WR 238605 against mefloquine resistant strain of parasite.

Day 3-6 Treatment

Additional studies using WR 238605 in therapeutic schedule i.e. day 3-6 post-infection using the same multi-resistant strain also shows no significant resistant reversal effect since the mean survival time of the mefloquine alone at different dose was 26.0, 31.50 and 37.66 days respectively which were longer as compared to corresponding combination treatment groups (WR 238605 + mefloquine), the MST being 21.5, 35.33 and 29.33 days). Mefloquine being a long acting compound provides prolonged suppression of blood parasitaemia. Slightly better suppression of parasitaemia on day 7 in WR 238605 + mefloquine groups, as compared to mefloquine alone groups, suggests some transient additive action of the two compounds (Table 30).

EVALUATION OF QUINIDINE FOR CHLOROQUINE RESISTANCE REVERSAL EFFECT

Day 3 to 6 treatment

Quinidine which is known to be effective against chloroquine resistant *P. falciparum*, was evaluated for possible additive antimalarial or resistance reversal effect in combination with chloroquine using multiresistant *P. yoelii nigeriensis* rodent strain. Results of the experiments in which therapeutic treatment with quinidine alone, chloroquine alone and combination of both quinidine with chloroquine were given from day 3-6 post-infection when the initial parasitaemia was in range of 2.5% are given in Table 31.

Analysis of results on day 10 post-treatment suggests a significant decrease of parasitaemia in group given quinidine and chloroquine combination (0.33 ± 0.08) in comparison to quinidine alone (5.95 ± 0.15) and chloroquine alone (5.2 ± 1.5). However, overall assessment of the data on mean survival time basis show that the

chloroquine treated group of mice survived for 11.55 days, quinidine alone group showed 11.83 days and chloroquine and quinidine groups survived for 24.16±9.08 to 27.0 days, suggesting the extension of mean survival time in the combination group. Overall data suggest that quinidine in combination with chloroquine exerts possibly resistance reversal effect since 4 out of 6 mice survived in quinidine + chloroquine combination groups but there was no survival in quinidine or chloroquine treated groups (Table 31).

B. Resistance reversal studies with mosquito transmissible *P. yoelii nigeriensis* (N-67) in Swiss mice

a) Selection of resistant strains

Four drug resistant strains showing resistance to chloroquine (128 mg/kg), mefloquine (128 mg/kg), halofantrine (128 mg/kg) and pyrimethamine (48 mg/kg) were selected after exposing the parent drug sensitive parasites to interrupted subcurative therapy with the respective antimalarials (Table 32). The stability of resistance was confirmed after transmission through the vector *An. stephensi*. These strains have been used for resistance reversal studies using i) Verapamil, ii) Amitryptline and (iii) Cyproheptadine.

b) Resistant reversal studies with chloroquine resistant strain

Combination of chloroquine (16 mg/kg) and verapamil (50 mg/kg) showed marked reduction in parasitaemia on day 4 compared to the chloroquine alone or verapamil alone treated groups. The combination has only transient suppressive effect observed one day after the last dose, while there was no significant difference in the parasitaemia in the combination and chloroquine alone treated groups after day 7 (Table 33). Antidepressant drug amitryptline in combination with chloroquine showed significant suppression of parasitaemia on day 4 and 7 compared to the corresponding controls, showing a transient suppressive efficacy of the combination (Table 33).

Resistance reversal studies with combination of cyproheptadine and chloroquine showed that the animals treated with combination of chloroquine (16 mg/kg) and cyproheptadine (10 mg/kg) were completely protected upto day 28 observation (Table 33).

c) Resistance reversal studies with mefloquine resistant strain

Studies using cyproheptadine in combination with mefloquine against mefloquine resistant strain showed significant activity of the combination. 70% of the combination treated animals did not develop any parasitaemia during the observation period while only transient low level parasitaemia was observed in the remaining 30% animals (Table 34). The resistance reversal potential of cyproheptadine warrants further evaluation in the primate malaria model.

Combination of mefloquine 8 mg/kg with amitryptline (50 mg/kg) showed significant suppression of parasitaemia on day 4 and 7 while mefloquine plus verapamil combination produced only transient suppression of parasitaemia compared to the corresponding controls (Table 34).

d) Resistance reversal studies with Halofantrine resistant strain

Halofantrine (4 mg/kg) in combination with cyproheptadine (10 mg/kg) protected all the treated mice during observation period of 28 days while partial reversal effect was observed with verapamil or amitryptline combinations (Table 35).

e) Resistance reversal studies with Pyrimethamine resistant strain

Combination of pyrimethamine 4 mg/kg with cyproheptadine (10 mg/kg) or with amitryptline (50 mg/kg) did not show any significant variation of parasitaemia from the group treated with pyrimethamine alone (Table 36).

C. Resistance reversal studies in simian malaria model/*P. knowlesi* rhesus monkey

Plasmodium knowlesi infection in rhesus monkey has been found to possess

innate resistance to mefloquine and the parasite has been found to show recrudescene even after 80 mg/kg x 3 days treatment. This simian model has been used for evaluating the resistance reversal efficacy of amitryptline and cyproheptadine.

Amitryptline

Two monkeys were inoculated with $1x10^6$ *P. knowlesi* blood stage parasites and when parasitaemia reached between 2-3%, the monkeys were treated with 20 mg/kg mefloquine x3 days plus 20 mg/kg amitryptiline x 5 days. The parasite clearance was observed in 48 hours; however, both the monkeys showed recrudescene on day 11 and 13 after the last dose of mefloquine (Fig. 10) indicating no resistance reversal action of amitryptiline. Mefloquine (20 mg/kgx3 days) alone showed recrudescence on day 9 (Fig. 11).

Cyproheptadine

P. knowlesi infected monkeys at (0.3-3.7 %) were administered 20 mg/kg mefloquine x 3 days plus cyproheptadine (0.6-10 mg/kg) x 5 days (Table 37). The parasitaemia clearance was recorded within 48-72 hrs. Subsequent observation up to day 60 did not show any recrudescence in any of the monkeys treated with mefloquine plus 10 or 5 mg/kg cyproheptadine while partial protection was recorded with lower doses of cyproheptadine. One monkey was treated with 10 mg/kgx5 day cyproheptadine alone, and this dose cleared the parasitaemia in 72 hrs, though there was recrudescence after 3 days (Table 37).

D. Studies on mechanism of resistance reversal

Several models and working hypothesis for the mechanism of resistance and resistance reversal have been proposed. In this context the most recent findings that the cytochrome P-450 (Cyt. P-450) dependent hydroxylase activities are higher in CQ resistant than in sensitive strain are of significance. In eukaryotic cells these mono-oxygenase systems of which cyt. P-450 is the terminal oxidase, are responsible for the metabolism of a wide variety of structurally unrelated

xenobiotics, including antimalarial drugs and endogenous compounds. In the present investigation we will characterize the cyt. P-450 system in malarial parasite. The method for biochemical localization of cyt. P-450 in the microsomal fraction of *P. knowlesi* has been standardized and the cyt. P-450 has been partially purified. It is presumed that the drug resistant parasites would show increased level of specific activity of cyt. P-450 enzyme in comparison to the sensitive counterpart. Further, it is believed that the resistance reversal agents such as verapamil and nifedipin etc. would tend to down regulate the P-450 levels of the drug resistant *Plasmodia*. It is proposed to test this hypothesis in a multidrug resistant rodent malaria (*P. yoelii*) model which shows high level of resistance to chloroquine, mefloquine and quinine and the reversal agents would be administered for 4-7 days.

11. *IN VITRO* CULTIVATION AND BIOASSAY FOR ANTIMALARIALS

A. *In vitro* cultivation of simian malaria parasite (*P. knowlesi*)

Studies have been carried out to standardize culture conditions for short as well as long term *in vitro* maintenance of simian malaria parasite *P. knowlesi*. The parasites were maintained in RPMI 1640 medium supplemented with 10% normal monkey serum using candle jar technique. Infected blood at 2% parasitaemia was collected aseptically in citrate saline. Infected blood was washed with incomplete medium and finally 6% haematocrit was prepared in complete medium and dispensed 3 ml in petri dishes or glass vials. The medium was changed at every 24 hours and thin smears were prepared to monitor the growth of the parasites.

Preparation of media

RPMI-1640	10.4 gm
HEPES buffer	5.94 gm
Gentamycin	40.0 mg
Distilled water	900 ml

The contents were dissolved and adjusted to 960 ml, sterilized by filtering the medium through 0.22 μm millipore filter and dispensed in 100 ml volumes in sterile screw cap bottles for storage.

Sodium bicarbonate solution (5%)

$NaHCO_3$ anhydrous	5.0 gm
Distilled water	100 ml

Dissolved and sterilized by millipore filtration and stored in screw-cap tubes in 5 ml aliquotes.

Normal monkeys serum (NMS)

Fresh blood was collected from normal monkeys by venous puncture and allowed to clot at room temperature for 30 minutes. After storage at 0°C overnight the serum was collected and dispensed into sterile tubes. Serum was inactivated at 56°C for 30 minutes.

Complete medium

4.2 ml of 5% $NaHCO_3$ was added to 95.8 ml of the incomplete medium. Finally 10 ml NMS was mixed with 90 ml of the above medium.

The growth of *P. knowlesi* was good in medium supplemented with 10% NMS. Nearly 60-70% of the parasite matured into schizont stage in 24 hours. Invasion into new erythrocytes was observed for five-six cycles. *P. cynomolgi* cultured in medium supplemented with 10% NMS showed the parasite maturation from ring to schizont in 48 hours but invasion rate was very low. However, in medium supplemented with 20% NMS the growth of parasite was better and parasites were maintained upto day 15.

B. Standardization of short term culture for *in vitro* drug assay

Disposable petridishes, 24 well culture plates and 96 well micro-culture plates were used for short term culture of *P. knowlesi* to assess their suitability for *in vitro* drug screening. The results have shown that the petridishes, 24 well

culture plates as well as 96 well micro-culture plates support the growth of parasite and cultures initiated at ring stage mature into schizont stage in 20-24 hours as monitored by Giemsa stained blood smears prepared at varying time intervals.

Assessment of parasite growth *in vitro* by use of radiolabelled precursors

Different radiolabelled nucleotides and aminoacids have been used to measure parasite growth and study the inhibitory effects of drugs on the growth of the parasite. Optimum concentration of radiolabelled precursors and drug dilutions were added to the micro-cultures and the plates were further incubated for 18 hours. After incubation, the labelled parasites were harvested into the glass fiber filters using glass distilled water and an automated multiple sample harvester. The filter paper discs were added to 10 ml of scintillation fluid and counts recorded in a liquid scintillation counter. Results were expressed as disintegration per minute (DPM).

Comparison of uptake of different labelled precursors

Comparative uptake of ^{3}H labelled thymidine, leucine, isoleucine and hypoxanthine was determined during the growth of *P. knowlesi in vitro*. Labelled precursors (0.5 μCi) were added into the culture wells at 0-3 hr and plates were further incubated for 18 hours at 37°C in candle jar. After incubation cells were harvested in cell harvester. The filter paper disc was dried and placed in scintillation vial and counts recorded in scintillation counter. Results showed that ^{3}H thymidine, leucine and isoleucine incorporation was very low as compared to ^{3}H hypoxanthine uptake (Table 38). Hence hypoxanthine was selected as the most suitable radiolabelled compound for *in vitro* drug assay studies. Webster and others (1981) have demonstrated that hypoxanthine is the major purine base utilized by the malaria parasite for synthesis of adenosine and guanine nucleotides and nucleic acids. The radio activity measured represents primarily ^{3}H hypoxanthine incorporation into the parasite. Background ^{3}H hypoxanthine incorporation by uninfected RBC's was low since these cells synthesize neither RNA nor DNA.

Standardization of optimum concentration of ^{3}H hypoxanthine for drug assay studies

P. knowlesi synchronized at ring stage was cultured in 96 well culture plates and radioactive counts after addition of 0.125, 0.25 and 0.5 μCi ^{3}H hypoxanthine were recorded after 18 hours incubation at 37°C in candle jar. Results showed that significantly high uptake of hypoxanthine was recorded with 0.5 uCi/well at parasitaemia levels rainging between 1-11% and ws found to be optimum for use in monitoring the growth *in vitro*. The uptake of hypoxanthine by uninfected cells was very insignificant (Table 39). Microscopic observations of Giemsa stained smears from cultures incubated under similar conditions (except addition of ^{3}H hypoxanthine) showed that the most of the ring stage parasites had matured into the trophozoites and schizonts.

Effect of duration of incubation on uptake of ^{3}H hypoxanthine

P. knowlesi synchronized at ring stage was cultured in 96 well micro-culture plates and incubated at 37°C in candle jar. ^{3}H hypoxanthine (0.5 uCi in 20 μl medium) was added to each micro-culture. Comparison was made of the quantitative uptake of labelled hypoxanthine after 4 hr and 24 hr incubation using variable percent parasitaemia and haematocrit. Results showed that uptake was very low during initial 4 hours (i.e. during the maturation of rings into early trophozoites), while significant incorporation was observed after 24 hours of culture i.e. during the period of schizont maturation both at high (9%) and low (1%) parasitaemia levels (Table 40).

Effect of parasite number and haematocrit on hypoxanthine incorporation

Comparison was made of the uptake of hypoxanthine at parasitaemia levels of 9, 3 and 1% and normal red blood cells (NRBC), as well as at different haematocrit viz. 6%, 3%, 1.5% and 0.75%. Synchronized *P. knowlesi* at ring stage were cultured in micro-culture plates. Micro-cultures were pulsed with 0.5

uCi ^{3}H hypoxanthine for 24 hours to re-record the radio isotope incorporation. Results in Table 41 show that incorporation of ^{3}H hypoxanthine was directly proportional to the increase in parasitaemia from 1 to 9%. At 6% haematocrit, the uptake was proportional to increase in parasitaemia from 1 to 3% since at high parasitaemia (9%) their was decline in the DPM values. A comparison of the results on parasitaemia versus haematocrit basis showed that the uptake of ^{3}H hypoxanthine at high parasitaemia (9%) was inversely proportional to haematocrit concentration i.e. there was increase in uptake with corresponding decline in the haematocrit. On the other hand, at low parasitaemia of 1%, this relationship was direct i.e. increase in haematocrit resulted in increase in uptake of radioactive precursors. At medium parasitaemia level (3%), the uptake of hypoxanthine was more or less identical with all haematocrit levels used in the study. In the uninfected cells the counts were very low and nearly identical at all the haematocrit levels.

***In vitro* antimalarial screening model: Evaluation of dose response of chloroquine using ^{3}H hypoxanthine incorporation**

Limited studies have been conducted to determine the dose response of chloroquine. *P. knowlesi* synchronized at ring stage with 6% parasitaemia and 1.5% haematocrit were incubated with different concentration of chloroquine (0.00015 μg/ml-10.0 μg/ml) in 96 well micro-culture plates and incubated at 37°C in candle jar. Micro-culture were pulsed with 0.5 μCi ^{3}H hypoxanthine after four hours and further incubated for 18 hours. Micro-culture plates were harvested after incubation. The filter paper discs were added to scintillation fluid and activity counted in scintillation counter. Data was analysed for determination of IC50/IC90 values and results are presented in Table 42.

B. *In vitro* cultivation of *P. falciparum*

Technology for *in vitro* cultivation of *Plasmodium falciparum* strains has been established. The parasite was cultured in medium RPMI-1640 supplemented

with 2% glucose and 10% O + human serum. Subcultures were done with human 0^+ erythrocytes. *P. falciparum* strains (NF54, FID3, FCD3) have been successfully maintained *in vitro*.

C. *In vitro* testing for tissue schizontocidal action

A method is being standardized for primary screening of prospective tissue schizontocides using *P. cynomolgi* exoerythrocytic stages cultured in rhesus hepatocytes. Assay was standardized using standard tissue schizontocidal drug primaquine. The drug was added 24 hrs after sporozoite invasion of cultures. Primaquine exerted tissue schizontocidal action against the primary EE stages of the parasite at concentrations as low as 0.1 μg/ml. Simultaneous experiments showed that chloroquine did not exert any parasiticidal effect even at concentrations of 5 μg/ml.

This assay will be useful for primary screening of tissue schizontocides and will go a long way to replace the costly *in vivo* rhesus monkey model for conducting large scale evaluation of potential tissue schizontocides. This study will also provide new leads for identification of the site of action of tissue schizontocides.

D. Standardization of *in vitro* antimalarial assays system using parasite LDH

The development of *in vitro* antimalarial screening of potential antimalarial as well as establishment of new assay systems to detect drug resistance character of the malaria parasite is receiving high priority in the collaborative programme. So far, the identification of resistance is generally done in the *in vitro* model by giving four doses of drug and recording the level of infection/% suppression of parasiaemia of the drug treated animals as compared to the untreated controls. The parasite like *P. yoelii nigeriensis* MDR strain can tolerate high level of antimalarials *in vivo* and has shown resistance to 128 mg/kg x 4 days chloroquine, 128 mg/kg x 4 days mefloquine and 400 mg/kg quinine x 4 days.

This MDR parasite is now being used to establish an *in vitro* system for detection of drug resistance based on possible inhibition of LDH activity of the parasite in presence of drugs.

Two assay systems have been initially investigated for detection of parasite LDH.

1. Reaction mixture containing Tris-Lactate Buffer (52 mM) B NAD (172 mM), NBT (0.24 mM), MTT (0.033 mM).

2. Reaction mixture containing Tris-Lactate Buffer (52 mM), APAD (172 mM), NBT (0.24 mM), MT (0.033 mM).

APAD cofactor containing the reaction mixture has shown a very high level of parasite activity even at 10-15% parasitaemia in comparison to the normal blood (control) which shows a very low level of activity.

Fig. 12 (infected blood versus normal blood) shows high sensitivity of APAD for LDH parasite quantitation using Sherman method.

The LDH detection system with APAD as co-factor can be developed to establish the *in vitro* system for antimalarial screening.

12. *IN VITRO* METHOD FOR EVALUATING METHEMOGLOBIN TOXICITY OF 8-AMINOQUINOLINES

There is a major emphasis in the project on developing *in vitro* protocols for comparative evaluation of methemoglobin toxicity of potential 8-aminoquinoline agents. The protocol is being standardized using primaquine as the reference drug. For MetHb *in vitro* assay mastomys erythrocytes were incubated with varying concentrations of the drug for 90 minutes. Methemoglobin formed was recorded at 630 nm and percentage was calculated with reference to the total hemoglobin in the lysate.

Standard compound N_aNO_2 was also used as the reference to standardize the test, since it is known to convert Hb to MetHb in 15-20 minutes. The results show

linear increase in MetHb formed at concentration between 10 to 1000 μm. The chloroquine used as reference negative drug did not produce appreciable MetHb, while reference 8-aminoquinoline drug primaquine produced MetHb. 3-4.5% at 10 μm, 8.0-11.6% at 100 μm and 23.8-29.6% at 1000 μm concentration. MetHb formed with 4-methyl primaquine at 100 μm was twice the values obtained with primaquine at the same concentration (Table 43).

13. PROPHYLACTIC STUDIES WITH RECOMBINANT IL-12 AGAINST SPOROZOITE INDUCED *P. CYNOMOLGI* B INFECTION IN RHESUS MONKEYS (SPONSORED BY U.S. NAVAL MEDICAL RESEARCH INSTITUTE)

Experimental Procedures

Course of *P. cynomolgi* infection in rhesus monkeys: Intravenous injection of *P. cynomolgi* sporozoites results in universal blood stage infection about 10 days later (range 8-12 days). *P. cynomolgi* is a relapsing malaria, similar to human vivax malaria. Relapses generally occur 10-15 days after clearance of blood-stage infection. In spleen intact animals, parasitaemia ranges from 3-8%. Parasitaemias are approximately twice as high in splenectomized animals. The infection in rhesus monkeys is generally self-limited and the monkeys exhibit no overt distress; they eat and drink normally at all levels of parasitaemia. Mortality does occur, generally in splenectomized animals with high parasitaemias. Analgesics are generally not required. Infected animals can be cured with chloroquine 5 mg/kg and primaquine 1 mg/kg for 7 days. Rhesus monkeys weigh approximately 5 kg.

(i) Determination of protective dose and schedule of IL-12 in prophylactic test

Five groups of 4 monkeys have been tested. The formulation was delivered subcutaneously in the nuchal region. rHu IL-12 was diluted in sterile 1% normal monkey serum in PBS (pH 7.2) to give required doses of rHu IL-12 in 1.0 ml.

Control monkeys were given 1.0 ml. 1% monkey serum in PBS (pH 7.2). rHU IL-12 was given to the following regimens:

Group	IL-12 dose	Treatment duration	No. of monkeys
1.	100 ng/kg	Day-2 to +10(alternate day)	4
2.	1 μg/kg	Day-2 to +10 (alternate day)	4
3.	10 μg/kg	Day-2 (Single dose)	4
4.	20 μg/kg	Day-2 and 0	2
5.	Control (vehicle)	Day-2 to +10 (alternate day)	4

Revalidation of IL-12 efficacy

1.	10 μg/kg	Day-2 (Single dose)
2.	Control (Vehicle)	Day-2 (Single dose)

IL-12 prophylactic efficacy was revalidated in an additional experiment consisting of one study group (3 monkeys) and one control group (1 monkey).

Background

Testing to date has proven rHu IL-12 safe in monkeys. The above doses were recommended by researchers of Hoffman-LaRoche who have performed several rHu IL-12 studies in both rhesus and *Siamiri scirurus* monkeys. In Siamiri monkeys the above doses were bioactive and safe, with no clinical abnormalities or serious toxicity. Hematologic and serum chemistry abnormalities included mild to moderate anemia and leukocytosis, hypoproteinemia, hypoalbuminemia, hypophosphatemia, and hypocalcemia. Their findings suggest that the above doses might be active and not cause serious adverse effect. Earlier work in mice at

Naval Medical Research Institute with higher IL-12 per weight doses did not show adverse effects.

Sporozoite challenge

One day 0 monkeys were injected in the mid-saphanous vein (using a 25 g needle and 3 ml syringe) with 10,000 sporozoites which had been dissected from the salivary glands of *Anopheles stephensi* mosquitoes fed on monkeys infected with *P. cynomolgi*. Beginning on day 7 after infection and continuing for 8 weeks, monkeys were bled from the marginal ear vein (approximately 20 μl by sterile lancet skin prick) to assess parasitaemia by Giemsa-stained blood smear. Smears were performed daily for the first 3 weeks and twice per week for an additional 5 weeks. Obtaining the blood sample does not require anesthesia and lasts less than one minute. Any discomfort felt by the animal is transitory. Prior to puncture the skin is swabbed with alcohol. All monkeys that developed parasitaemia were cured with chloroquine 5 mg/kg and primaquine 1 mg/kg for 7 days by oral catheter.

Human rIL-12 used in this study was produced in *E. coli* and provided by F. Hoffman-LaRoche, Nutely, NJ. On every other day dose was used because of the prolonged half-life of rHu IL-12 in monkeys (14 hours) compared to mouse IL-12 in mice (3 hours).

Results

The four vehicle control monkeys (Group 5) developed patent infection between day 10-12 post sporozoite challenge. Likewise four monkeys each in group 1 (100 ng/kg dose) and group 2 (1 μg/kg x dose) also developed patent infection between day 11-18 (Table 44). None of the 4 monkeys in Group 3 (10 μg/kg single dose) and 2 monkeys in Group 4 (20 μg/kg x 2 doses) developed patent infection up to observation period of 70 days post challenge. The results thus show prophylactic efficacy of r IL-12 at 10 μg/kg dose (Table 44).

In the revalidation experiment to confirm the protective dose, the vehicle control monkey became patent on day 10 while none of the 3 monkeys treated with 10

μg/kg dose developed patent infection till 70 days, post sporozoite inoculation and were protected (Table 45). The study shows very good prophylactic efficacy of Hu r-IL-12 against sporozoite induced *P. cynomolgi* B. Further studies on the length of prophylactic efficacy and the validation of minimum prophylactic dose would be useful.

Determination of cytokine levels after rHuIL-12 injection

As discussed above, the parasite killing effect of IL-12 appears to be mediated by IFN-r although this has not been assessed in the monkey model. To assess this relationship, we have determined serum levels of IFN-r and IL-12 and mRNA expression kinetics of IFN-r IL-6, IL-10, IL-12, IL-15 and TNF-α. Ten blood samples (approximately 3 ml each) were obtained from each monkey for determination of IFN-r levels; this included a baseline sample prior to rHu-iL-12 injection, alternate day samples from day 0 to day 10, and twice weekly samples from day 11 to day 25. Blood was drawn from the external saphenous vein of the leg using a 22 g needle and 5 ml syringe. Serum samples were separated and frozen for later transport to Dr. Ansari (CDC), Atlanta, for testing and quantitation of cytokines. The results showed that Group 3, administered 10 μg/kg single dose was the only group in which plasma r-Hu-IL-12 levels were above control limits. These levels peaked on day 0 and then dropped back to near baseline by day 4 (Fig. 13). Serum IFN-r levels in group 3 rose steadily after IL-12 administration, peaking on day 2 and returned to near baseline on day 11 (Fig. 14).

There was significant increase in IFN-r, IL-6, IL-10, IL-12 and IL-15, mRNA expression in monkeys that received r-Hu-IL-12 and the results are shown in Tables 46 and 47.

Determination of efect of rHu IL-12 on infectivity of gametocytes

Previous work at CDRI at the rhesus *P. cynomolgi* model has shown that IFN-r inhibits the ability of gametocytes to infect mosquitoes. This is determined by monitoring the number of oocysts which develop on the gut wall of mosquitoes

after feeding on gametocytemic monkeys. Gametocytes normally appear about 2 weeks after infection and mosquito oocyst counts over the following ten days range from 20-200. IFN-r given during the gametocytemic phase results in the complete absence of oocysts in mosquitoes (Dr. Renu Tripathi, CDRI, personal communication). We plan to test the hypothesis that r-Hu IL-12 will have the same effect due to its ability to stimulate IFN-r production. Two monkeys will be treated with rHu IL-12 (20 μg/kg in a single dose) at appropriate gametocytemia level and three to four day old *A. stephensi* mosquitoes will then be fed on the monkeys at 6, 24 and 48 hours after treatment. These mosquitoes will be maintained in the insectory and midgut dissections performed on day 7 or 8 to monitor oocyst number.

Table-1 : Serial cyclic passages of sporozoite induced p.cynomolgi B in rhesus monkeys since March, 1993.

Sporozoite passage no.	Date of inoculation	Monkey No.	Sporozoite inoculum (i.v.)	Day of patency
87	6.3.93	7666	$1.44X10^6$	8
88	13.4.93	7679	$0.73X10^6$	9
89	20.5.93	7680	$1.64X10^6$	8
90	26.6.93	7775	$1.24X10^6$	8
91	5.8.93	7782	$0.70X0^6$	9
92	8.10.93	7827	$0.76X10^6$	9
93	1.12.93	7850	$1.14X10^6$	8
94	10.1.94	7831	$0.96X10^6$	8
95	14.2.94	7911	$1.54X10^6$	8
96	18.3.94	8018	$0.83X10^6$	9
97	29.4.94	8029	$1.14X10^6$	8
98	17.6.94	8084	$1.40X10^6$	8
99	5.8.94	8086	$0.72X10^6$	9
100	10.9.94	8179	$1.15X10^6$	8
101	28.10.94	8258	$0.86X10^6$	9
102	22.12.94	8240	$1.24X10^6$	8
103	10.2.95	8299	$0.75X10^6$	9
104	21.3.95	8307	$0.50X10^6$	9
105	26.5.95	8348	$0.30X10^6$	9
106	8.7.95	8405	$1.20X10^4$	10
107	25.7.95	8367	$0.22X10^6$	9
108	28.8.95	8426	$3.00X10^4$	10

109	5.10.95	8437	$1.40X10^{4}$	11
110	7.12.95	8467	$1.00X10^{5}$	9
111	18.1.96	8471	$0.80X10^{6}$	8
112	27.2.96	8556	$0.75X10^{6}$	9
113	3.4.96	8577	$1.30X10^{6}$	8
114	30.5.96	8607	$0.80X10^{6}$	8
115	11.7.96	8605	$0.64X10^{6}$	9
116	20.8.96	8616	$1.20X10^{6}$	8
117	1.10.96	8637	$0.75X10^{6}$	8
118	4.11.96	8701	$1.00X10^{6}$	9
119	26.12.96	8783	$0.50X10^{6}$	9
120	29.1.97	8784	$0.26X10^{6}$	9

Table- 2

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Chloroquine (3 dose regimen)

BN: AU 29291

DATE REC'D: Oct.1993

QUANTITIY: 500 gm

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.= 518

Base= 320

BLOOD SCHIZONTOCIDAL TEST (X 3 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
5.0	8083	Recrudescence on day 16
5.0	8088	Recrudescence on day 14
7.5	8035	Cured
7.5	8074	Recrudescence on day 18
10.0	8078	Cured
10.0	7992	Cured
7.5*	8083	Cured
7.5*	8088	Cured
10.0*	8074	Cured

* Monkeys retreated after recrudescence at the lower dose.

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose validation in Sp. passage 90)

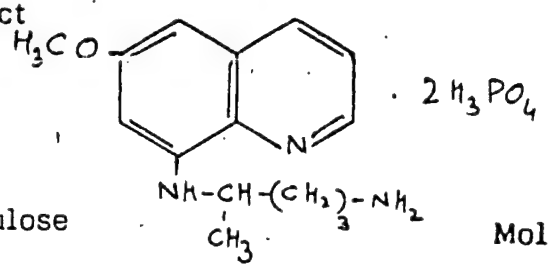
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl Cellulose

ROUTE Oral



Mol.Wt.= 455

Base= 259

PROPHYLACTIC TEST (X 3 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1,78	7772	Cured
1.78	7776	Cured
Control		
-	7775	Patent on day 8
-	7773	Patent on day 9

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose revalidation in serial Sp. Passage 86)

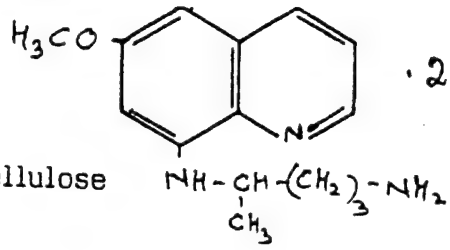
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl cellulose

ROUTE Oral



Mol.Wt.= 455

Base= 259

RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.00	7552	Cured
1.00	7556	Cured
0.316	7548	Relapse on day 29
0.316	7560	Relapse on day 37
Chloroquine Control		
-	7558	Relapse on day 16
-	7578	Relapse on day 19

TABLE - 5

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose revalidation in serial Sp Passage 90)

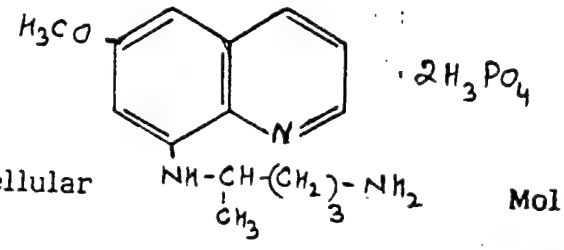
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl Cellular

ROUTE Oral



Mol.Wt.= 455

Base= 259

RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.00	7773	Cured
1.00	7774	Cured
Chloroquine Control	7768	Relapse on day 9

Table- 6

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 142490 (Mefloquine)

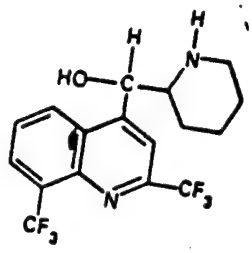
BN: BE. 16387

DATE REC'D: Nov. 1993

QUANTITIY: 1000 mg

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.= 414.5

Base= 378

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7904	3.16	No parasite clearance
7911	3.16	No parasite clearance
7906	10.0	Cured
7909	10.0	Cured
7903	31.6	Cured
7908	31.6	Cured

Table-7

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Mefloquine

BN: BE 19191

DATE REC'D: July 1994

QUANTITIY: 85 gm

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.= 414.5

Base= 378

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
10.0	8140	• Cured
10.0	8141	Cured

TABLE-8

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 171669 (Halofantrine)

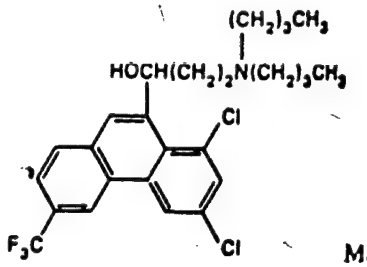
BN: BK 64002

DATE REC'D: Oct. 1988

QUANTITIY: 5 gm

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.=

Base=

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7912	3.16	Recrudescence on day 12
7921	3.16	Recrudescence on day 14
7919	10.0	Cured
7924	10.0	Cured
7902	31.6	Cured
7905	31.6	Cured

Table- 9

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Halofantrine

BN: BK 64002

DATE REC'D: July 1994

QUANTITIY: 50gm

VEHICLE: Aqueous

ROUTE Oral Mol.Wt.=

Base=

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
10.0	8080	Cured
10.0	8081	Cured

Table- 10

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Halofantrine

BN: BK 64002

DATE REC'D: July 1994

QUANTITIY: 50 gm

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

Expt.II I BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
5.62	8139	Recrudescence on day 19
5.62	8180	Cured
5.62	8265	Cured
5.62	8273	Cured
10.0	8138	Cured
10.0	8181	Cured

Table-11

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 242511

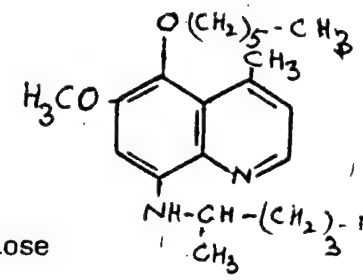
BN: BL 09412

DATE REC'D:

QUANTITIY: 2 gm

VEHICLE: Methyl cellulose

ROUTE Oral



Mol.Wt.= 571

Base= 373

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7907	0.316	Recrudescence on day 16
7910	0.316	Recrudescence on day 16
7913	1.0	cured
7923	1.0	Cured
7918	3.16	Cured
7922	3.16	Cured

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 242511

BN: BL 09412

DATE REC'D:

QUANTITIY: 2 gm

VEHICLE: Methyl Cellulose Mol.Wt.= 571

ROUTE Oral Base= 373

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.0	8075	Cured
1.0	8079	Cured

TABLE - 13

Comparison of the blood schizontocidal activity of WR 238605 and primaquine against trophozoite induced P.cynomolgi B and P. fragile in rhesus monkeys.

Dose mg(base)/ kgx7 days	Total course dose mg(base)/kg	No. of monkeys treated	Response to treatment	
			No. of monkeys cured	No.of monkeys showing recrudescence(on day)
A. Plasmodium cynomolgi B Infection				
WR238605				
0.316	2.21	4	0	4 (7,13,18,20)
1.00	7.00	12	10	2 (20,26)
3.16	22.12	6	6	0
Primaquine				
1.00	7.00	2	0	2 (10,12)
3.16	22.12	4	0	4 (13,15,16,19)
10.00	70.00	4	1	3 (15,24,28)
B. Plasmodium fragile infection				
WR 238605				
0.316	2.21	4	0	4 (16,19,24,28)
1.00	7.0	11	10	1 (36)
3.16	22.12	4	4	0
Primaquine				
1.00	7.00	2	0	2 (13,16)
3.16	22.12	3	1	2 (17,20)
10.00	70.00	3	2	1 (18)

TABLE- 14

Gametocytocidal activity of compound WR 238605 in the *P. cynomolgi* - *A. stephensi* - rhesus monkey model

DOSE (Mg/Kg) AT 0 Hr.	TIME OF MOSQUITO FEEDING	PARASITAEMIA/MM^3 ASEXUAL	PARASITAEMIA/MM^3 GAMETO-CYTAEMIA	DAY 7 OOCYST RECORD NO. OF MOSQUITO INFECTED/ DISSECTED (% INFECTI-VITY)	DAY 7 OOCYST RECORD OOCYST NUMBER (MEAN± SD)
1.00	-1Hr.	23112	749	32/37 (86.49%)	33.16±22.18
	+6Hr.	-	-	31/36 (86.11%)	42.26±23.76
	+24Hr.	26215	533	31/39 (79.49)	25.77±17.63
	+48Hr.	7383	321	0/30 (Nil)	-
1.00	-1Hr.	39055	1391	32/40 (80.0)	17.13±10.01
	+6Hr.	-	-	32/44 (72.73%)	13.69±7.20
	+24Hr.	28248	321	-0/31 (Nil)	-
	+48Hr.	5992	107	0/23 (Nil)	-
1.00	-1Hr.	48384	6832	25/34 (73.53%)	32.12±13.62
	+6Hr.	-	-	32/40 (80.0%)	31.06±12.73
	+24Hr.	42448	3256	36/48 (75.0%)	30.86±13.81
	+48Hr.	20832	1008	0/25 (Nil)	-
2.00	-1Hr.	54805	2938	43/53 (81.13%)	28.91±18.43
	+6Hr.	-	-	47/58 (81.03%)	27.13±16.30
	+24Hr.	26555	1243	0/38 (Nil)	-
2.00	-1Hr.	42619	2398	25/39 (64.10%)	18.40±10.19
	+6Hr.	-	-	30/48 (62.5%)	17.83±6.80
	+24Hr.	26487	1199	0/36 (Nil)	-
2.00	-1Hr.	42036	3503	22/27 (81.48%)	35.32±13.34
	+6Hr.	-	-	28/34 (82.35%)	26.89±11.19
	+24Hr.	21344	2668	0/31 (Nil)	-
4.00	-1Hr.	73902	3390	28/33 (84.85%)	29.75±12.41
	+6Hr.	-	-	31/36 (86.11%)	38.16±19.28
	+24Hr.	47008	1808	0/30 (Nil)	-

Table-15

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 (Shorter 3 dose regimen)

BN: BK 73252

DATE REC'D: July 1994

QUANTITIY: 10 gm

VEHICLE: Methyl Cellulose

ROUTE Oral

Mol.Wt.= 531

Base= 463

RADICAL CURATIVE TEST (X 3 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
Expt. I		
0.50	8142	Relapse on day 25
0.50	8144	Relapse on day 43
1.00	8076	Cured
1.00	8146	Cured
2.00	8054	Cured
2.00	8116	Cured
-	8077	Relapse on day 30
Expt. II		
0.75	8424	Cured
0.75	8427	Cured
0.75	8433	Cured
Chloroquine Control	8432	Recrudescence on day 16

Monkeys were concurrently administered chloroquine @ 10.0 mg(base)/kg X 3 days.

Table-16

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 238605 + Halofantrine combination

BN: BK 73252 , BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.=

Base=

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
WR 238605 + Halofantrine		
0.316 + 1.00	8264	Recrudescence on day 23
0.316 + 1.00	8275	Recrudescence on day 20
0.316 + 3.16	8274	Cured
0.316 + 3.16	8276	Cured
0.316 + 5.62	8271	Cured
0.316 + 5.62	8272	Cured

Table- 17 : P.cynomolgi- Rhesus Monkey Model

Blood Schizontocidal Activity of Halofantrine and WR 238605 combination (Summarized data)

Treatment Regimen mg/kg x 7 days			No. of* monkeys treated	Response to treatment	
Halofantrine + WR 238605				Number** protected	Number Recrudesced (on day)
1.00	+	0.316	2	0	2 (20, 23)
3.16	+	0.316	2	2	-
5.62	+	0.316	2	2	-
10.00	+	-	4	4	-
5.62	+	-	4	3	1 (19)
3.16	+	-	2	-	2 (12, 14)
-	+	3.16	2	2	-

* Treatment administered orally once daily for seven consecutive days.

** Monkeys which did not show any recrudescence upto day 60 post treatment were recorded as protected.

Table- 18

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine combination

BN: BK 73252 + BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

Expt.1. RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)			MONKEY NO.	RESULT
WR 238605 + Halofantrine				
0.316	+	3.16	8143	Cured
0.316	+	3.16	8149	Cured
0.316	+	5.62	8145	Cured
0.316	+	5.62	8147	Cured
0.316	+	10.0	8114	Cured
0.316	+	10.0	8115	Cured

Table- 19

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPORozoite INDUCED TEST

COMPD: WR 238605 + Halofantrine

BN:

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

RADICAL CURATIVE TEST (X 7 day)

Expt.II

DOSE mg/kg(base)			MONKEY NO.	RESULT
WR 238605	+	Halofantrine		
0.316	+	1.78	8243	Relapse on day 26
0.316	+	1.78	8244	Cured
0.316	+	3.16	8238	Cured
0.316	+	3.16	8241	Cured
0.316	+	5.62	8237	Cured
0.316	+	5.62	8242	Cured
0.10	+	10.0	8245	Relapse on day 13
0.10	+	10.0	8246	Relapse on day 15
0.316	+	-	8239	Relapse on day 26

Table- 19 (Contd.)

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine Combination.

BN: BK 73252 : BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.=

Base=

EXPT.III RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
WR 238605 + HAlofantrine		
0.316 + 3.16	8310	Cured
0.316 + 3.16	8315	Cured
0.10 + 10.00	8313	Cured
0.10 + 10.00	8316	Relapse on day 39
- 10.00	8303	Relapse on day 15
- 10.00	8305	Relapse on day 14

TABLE-20 : P. cynomolgi- Rhesus Model

Anti- Relapse Activity of Halofantrine and WR 238605 combination.

Treatment Regimen mg/kg x 7 days			No. of* monkeys treated	Response to treatment	
Halofantrine	+	WR 238605		Number** protected	Number Relapsed (on day)
10.00	+	-	2	-	2 (14, 15)
-	+	0.316	1	-	1 (39)
10.00	+	0.316	2	2	-
5.62	+	0.316	4	4	-
3.16	+	0.316	6	6	-
1.78	+	0.316	2	1	1 (26)
10.00	+	0.10	4	1	3 (13, 15, 39)

* Treatment adminsitered orally (once daily) for seven consecutive days

** Monkeys that did not show any relapse upto day 90 post-treatment were recorded as protected.

Table- 21

COMPOUND	:	Mefloquine + WR 238605 Combination
BN	:	BE 16387/BK 73252
Date Received	:	Nov,93
Quantity	:	
Vehicle	:	Aqueous
Route	:	Oral

BLOOD SCHIZONTOCIDAL TEST X 7 DAY

Dose (mg/kg) WR 238605		Mefloquine	Monkey No.	Result
0.316	+	3.16	8341	Recrudescence on day 25
0.316	+	3.16	8344	Recrudescence on day 26
0.316	+	5.62	8343	Cured
0.316	+	5.62	8345	Cured
-		5.62	8340	Recrudescence on day 17
-		5.62	8342	Recrudescence on day 12

Table- 22

COMPOUND : Mefloquine +WR 238605 combination

BN : BE 16387/BK 73252

Date Received :

Quantity :

Vehicle : Aqueous

Route : Oral

RADICAL CURATIVE TEST (x7 DAYS)

Dose(mg/kg)			Monkey No.	Result
WR 238605	+	Mefloquine		
0.316		5.62	8394	Cured
0.316	+	5.62	8406	Cured
0.316	+	10.00	8400	Cured
0.316	+	10.00	8408	Cured
Control				
-		10.00	8392	Relapse on day 11
-		10.00	8413	Relapse on day 12

Table- 23 Blood schizontocidal activity of antihistaminic drugs against multiresistant P.yoelii nigeriensis (MDRI)

Inoculum : 1 x 10^5 parasitised RBC (i.p.)
Host : Swiss mice (20g+2g)
Treatment schedule : Day 0 to+3 (4 doses, oral)

Treatment (x 4 days)	Parasitaemia %													MST (Days)
	Day 4	Daya5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	Survi-val	
Terfenadine (Trexyl 60) 60mg/kg	9.6±2.5 ±1.44	19.6± ±3.18	67±2.82 ±2.00	92±0.0 ±0.0										7.0
Mebhydrolin (Incidal) 100mg/kg	6.5±7.68 3.84	27.25± 35.26 ±17.63	35.75± ±44.16 ±22.08	2.12± 2.65 ±1.88	14±5.65 +4.0	62.5± 3.53± ±2.50	80±0.0± 0.0							8.88
CDRI 73/602 (Antihistaminic Compound)	3.91±5.12 2.09	22.8± 29.3± 13.13	37.0± 34.2 ± 17.13	58.5± 1909± 13.54										6.83
Cyproheptadine (Ciplactin) 40mg/kg	Toxic	00.0± 00	00.0± 00	00.0± 00	00.0± 00	00.0± 00	0.2±0.0 ±00	1.5±0.0 ±00	5.0±0.0 ±00	46.0±0.0				
20mg/kg	0.0±0.0±	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	.08±0.17 ±.07	1.0±2.23 ±1.0	7.0±15.65 7.01	0.13±0.25 0.12	2.0±4.0 2.0	15.0±30.0 15.0	3/6	<16.0
10mg/kg	0.58± 1.4±0.57	3.75± 8.47± 3.47	12.13± 24.6± 10.08	9.64± 12.30 5.57	3.5± 5.60± 3.23	7.66± 6.65± 3.84	26.6± 27.2± 15.72	38.3± 30.9± 17.87						9.83
Control	11.5± 6.24± 3.12	57.5± 28.7± 14.36	63± 25.9± 14.9	83.5± 16.26± 11.53										7.25

Table- 24 Sequential maintainence of P.knowlesi for selection of chloroquine resistant strain by interrupted subcurative therapy.

No.	Monkey No..	Date of inoculation	Exposure to chloroquine			Isolate cryopreserved
			No. of doses	Duration (Days)	Total dose (mg/kg)	
Rh-1	7943	19.1.94	5 doses (0.5-3.0 mg/kg)	8 days	7mg/kg	
Rh-2	7945	28.1.94	25 doses (.2-3 mg/kg)	82 days	23.7 mg/kg	R1- 2.4.94 R2- 26.4.94
Rh-3	8027	20.4.94	32 doses (.5-.2 mg/kg)	75 days	24.5 mg/kg	
Rh-4	8085	4.7.94	11 doses (.5-2 mg/kg)	44 days	10 mg/kg	
Rh-5	8087	17.8.94	4 doses (.5-2 mg/kg)	27 days	5.5 mg/kg	R3- 13.9.94
Rh-6	8282*	22.12.94	13 doses (1-2 mg/kg)	54 days (till 14.2.95)	16 mg/kg	

* Monkey inoculated with cryopreserved sample (R3) of 13.9.94.

Table- 25

Effec ts of different concentrations of Verapamil with chloroquine on reversal of drug resistance in P.yoelii nigeriensis strain

Strain: P.yoelii nigeriensis (multi drug resistant), Inoculum : $1x10^6$ parasites; Route: Oral; Treatment: 4 days (i.e. from day 0 - Day +3)

Drug	Dose (day)-+3)	Day 4 Mean±SD±SE	% Parasitaemia Record (Mean±SD) (No. of mice surviving)						
			Day 7	Day 14	Day 18	Day 21	Day 24	Day 28	MST
Control	-	9.38±4.7±1.9 (6)							6.0
Chloroquine	8mg/kg	Nil (8)	3.32±4.5 ±1.6 (8)	7.5±0.0	7.0±0.0				10.75
Verapamil	25mg/kg	18.7±11.6 (7)	32.9±15.9 (4)						8.4
Verapamil + Chloroquine	25mg/kg + + 8mg/kg	Nil (8)	0.3±0.5 ±0.2 (8)	8.6±10.5 ±5.3 (4)	3.3±4.7 ±3.3 (2)	0.2±0.3 ±0.2 (2)	Nil (1)	Nil (1)	12.25
Verapamil + Chloroquine	10mg/kg + + 8mg/kg	Nil (8)	3.7±9.8± ±3.5 (8)	5.2±2.9 ±2.1 (2)	8.0± (1)	0.4± (1)	-	-	12.63
Verapamil + Chloroquine	1.0mg/kg + 8mg/kg	Nil (8)	5.3±7.9 ±2.8 (9)						9.8
Verapamil + Chloroquine	0.5mg/kg + 8mg/kg	Nil (7)	1.4±3.5 ±2.5 (7)						9.13

Table- 26

Effects of different concentration of Verapamil with chloroquine on rversal of drug resistant
Strain: P.yoelii nigeriensis; Inoculum: $7x10^6$; Routeof drugs: Oral, Dose time; 4 days (day 3-6)

Drug	Dosemg/kg (day 3-6)	Day 4	& Parasitaemia (No. of mice surviving) Mean±AS) Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day	MST
Control Av. 2.5% of 25 mice		Nil (5)	2.68±0.8 ±0.6 (2)							7.4
Chloroquine	8	Nil (7)	0.3±0.8 ±0.3 (7)	6.3±3.8 ±1.5 (6)	4.65±4.5 ±2.2(4)	1.8±2.8 ±1.6 (4)	0.2±0.2 ±0.1 (4)	Nil (4)	Nil (3)	21.14
Chloroquine	16	Nil (7)	0.4±0.8 ±0.3 (7)	2.9±3.5 ±1.3 (7)	1.37±0.3 ±0.2 (3)	0.2±0.3 ±0.2 (3)	0.1±0.2 ±0.1 (3)	Nil (3)	Nil (3)	19.14
Verapamil	25	- (7)	17.5±8.9 (3)	20.0±0.0 (1)						8.3
Verapamil + Chloroquine	5	Nil (7)	0.14±0.4 ±0.14 (7)	8.5±6.8 ±2.6 (7)	2.8±4.4 ±1.8 (6)	1.23±2.8 ±1.2 (6)	Nil (6)	Nil (6)	(6)	25.75
Verapamil + Chloroquine	8	Nil (30)	Nil (3)	6.4±4.9 ±2.8 (3)	21.7±22.5 ±12.9 (3)	2.8±3.9 ±2.8 (2)	Nil (2)	Nil (2)	Nil (2)	24.60
Verapamil + Chloroquine	8	Nil (6)	Nil (6)	2.2±1.6 ±0.65(6)	4.8±7.3 ±3.3(5)	0.7±1.4 ±0.7(4)	Nil (4)	Nil (4)	Nil (4)	23.67
Verapamil + Chloroquine	8	Nil (6)	0.01±0.02 ±0.01 (7)	4.5±2.8 ±1.13 (6)	13.2±12.0 ±6.9 (3)	1.3±1.8 ±1.3 (2)	Nil (1)	Nil (1)	Nil (1)	15.71

Tabel- 27

Curative efficacy/chloroquine resistant reversal activity of nifedepin with chloroquine.

Drug	Dose	Drug schedule	Parasitaemia% Mean±SE (no. of mice surviving) Day 4	Day 7	Day 14	Day 18	Day 21	Day 28	MST
Nifidepine + Chloroquine	25mg/kg + 8mg/kg	3-7 days	(7)	0.5±0.5 (7)	7.35±1.79 (7)	1.75±1.03 (4)	Nil (3)	Nil (3)	20.9
Nifidepine + Chloroquine	15mg/kg + 8mg/kg	3-7	(7)	0.07 ±0.07	6.27 ±2.61	0.083 ±0.06	Nil (3)	Nil (3)	24.7
Nifidepine + Chloroquine	10mg/kg + 8mg/kg	3-7	(6)	Nil (6)	1.16±1.16 (6)	1.27±1.27 (6)	Nil (3)	Nil (3)	24.8
Nifidepine + Chloroquine	5mg/kg +	3-7	(7)	Nil (7)	2.20±2.20 (3)	5.6±0.0 (1)	-	-	14.4
Nifedepine	25mg/kg	3-7	(7)	36.25 ±13.29 (2)	-	-	-	-	7.1
Chloroquine	8mg/kg	3-7	(7)	0.29 ±0.28 (7)	4.67 ±2.23 (4)	1.8 ±1.60 (4)	0.15 ±0.09 (4)	Nil	21.14
Control	-	-	(5)	26.8±0.64 (2)					7.4

Table- 28

Evaluation of WR 238605 with chloroquine against *P.yoelii nigeriensis* (Multi Drug Resistant) for resistance Reversal study

Treatment schedule D o→D +3, Route of drug administration (Oral) Av. wt. of mice = 20 gm

Drug	Dose mg/kg	No. of mice	Mean of % parasitaemia on days							M.S.T.
			Day 4	Day 7	Day 10	Day 14	Day 21	Day 28	No. of mice survived	
Control	-	5	25.81 ± 3.2							6.2±.82
Chloroquine + WR 238605	8+0.5	5	0.75 ± .35	1.16 ± .52	1.45 ± .52	2.7 ± .98	-ve	-ve	2	19.4±9.0
Chloroquine + WR 238605	4+0.5	5	0.88 ± .10	.98 ± .20	1.8 ± .75	2.5 ± .70	2.5 ±0			14.4±5.9
Compound WR 238605	0.5	5	11.33 ±3.2							5.0± .83
Chloroquine	8.0	5	1.48 ±.48	2.0 ±.57	2.25 ±.25	2.55 ±.70	-ve	-ve	2	17.8±9.4
Chloroquine	4.0	5	2.44 ±.72	2.4 ±.73	4.33 ±.28	6.5 ±0				12.8±4.2

Table- 29

Evalaution of mefloquine with WR 238605 for study of drug reversal activity against P.yoelii nigeriensis(MDR)

Treatment schedule- D_0-D+3 Av.wt. of mice= 20 gm

Drug	Dose mg/kg	No.of mice	Mean of % parasitaemia on days				MST
			Day 4	Day 7	Day 10	Day 14	
Mefloquine	1.0	5	3.8	7.0	-	-	6.6
Mefloquine	2.0	5	3.7	4.5	4.5	-	9.2
Mefloquine	4.0	5	0.24	.58	.58	1.3	14.0
Mefloquine	8.0	5	0.24	3.45	.83	1.29	15.0
WR 238605	0.5	5	26.6	-	-	-	6.2
WR 238605+Mefloquine	0.5+1.0	5	1.8	5.0	-	-	6.6
WR 238605+Mefloquine	0.5+2.0	5	1.7	2.25	4.5	5.0	10.0
WR 238605+Mefloquine	0.5+4.0	5	1.06	1.25	1.58	2.2	11.0
WR 238605+Mefloquine	0.5+8.0	5	0.22	.32	.87	1.15	13.2
Control	-	5	27.6	-	-	-	5.8

Table 30

Evaluation of WR 238605 with mefloquine for drug reversal study against P.yoelii nigeriensis (multi drug resistant strain)

Treatment therapeutic (D+3 to D+6)

Drug	Dose mg/kg	No.of mice	% Parasitaemia on days						No.d mice survival on days(50)	MST (Days)
			3	4	7	10	14	35		
Control			6.7±2.3	16.66±3.2	-	-	-	-	-	5.0
WR 238605 + Mefloquine	0.5±16.0	6	6.0±1.5	1.5±	0.71±.20	1.05±.44	1.2±.44	.15±.06	2	29.33
WR 238605 + Mefloquine	0.5±8.0	6	4.7±.51	1.2±.59	0.65±.36	1.5±.58	.71±.64	0.36±.15	3	35.33
WR 238605 + Mefloquine	0.5±4.0	6	4.23±1.06	1.13±.32	0.73±.29	1.21±.27	2.1±.22	0	1	21.5
WR 238605	0.5	6	5.66±1.5	14.95±3.3	-	-	-	-	-	5.16
Mefloquine	16.0	6	5.72±1.5	1.25±.22	0.68±.82	1.75±.82	1.66±.42	0.22±.17	4	37.66
Mefloquine	8.8	6	4.66±1.3	1.63±.62	1.23±.28	2.08±.90	3.7±.90	0.36±.15	3	31.50
Mefloquine	4.0	6	4.53±1.7	1.68±.64	1.15±.31	1.33±.25	1.45±.41	0.30±0	1	26.0

Table-31

Evaluation of Quinidine with chloroquine against multi drug resistant P.yoelii nigerinsis

Treatment Therapeutic D+3 to D+6

Drug	Dose mg/kg	No.of mice	% Parasitaemia on days							MST (Days)
			3	4	7	10	14	30	No.of mice survival	
Control	-	6	2.58±.58	15.16±.98	-	-	-	-		7.16
Quinidine + Chloroquine	25+8	6	2.5±.40	3.25±.68	0.78±.36	0.33±.08	.76±.40	0	4	24.16±9.08
Quinidine + Chloroquine	25+4	6	2.58±.37	3.66±.40	.75±.20	0.95±.08	1.76±.79	0	4	27.0
Quinidine + Chloroquine	15+8	6	2.57±.49	4.08±1.1	.48±.11	0.91±.17	1.6±.5	0	4	23.833
Quinidine + Chloroquine	15+4	6	2.58±.36	4.53±.49	.71±.06	.75±.31	1.6±.05	0	4	25.33
Quinidine + Chloroquine	10+8	6	2.50±.40	4.58±.49	1.3±.36	.83±.20	0.92±.50	0	4	23.83
Quinidine + Chloroquine	10+4	6	2.33±.40	5.5±1.1	2.0±.15	5.2±.35		-	-	12.83
Quinidine	25.0	6	2.08±.25	5.25±.82	5.75±.15	5.95±.15	-	-	-	11.83
Quinidine	15.0	6	2.5±.40	4.2±1.3	1.0±.26	1.5±.05	1.25±.50	-	-	13.833
Quinidine	10.0	6	2.5±.40	5.9±1.6	4.95±.15	4.55±.50	-	-	-	10.0
Chloroquine	8.0	6	2.16±.25	5.5±.15	.88±.34	5.2±1.5	-	-	-	11.55
Chloroquine	4.0	6	2.33±.25	7.33±1.2	5.5±1.5	6.0±0	7.5±0	-	-	13.833

Table- 32 Establishment of drug resistant isolates of Plasmoidum yoelii nigeriensis (N-67) in Swiss mice model.

Isolate resistant to	Curative dose in parent strain (mg/kg x 4 days)	Resistance level (mg/kg x 4 day)	Stability after transmission through mosquitoes
Chloroquine	16 mg/kg	>128 mg/kg	Stable
Mefloquine	8 mg/kg	>128 mg/kg	Stable
Halofantrine	4 mg/kg	>128 mg/kg	Stable
Pyrimethamine	4 mg/kg	> 48 mg/kg	Stable

Table- 33 Resistance modulation studies against CHLOROQUINE RESISTANT isolates of P.yoelii nigeriensis (N-67)- Swiss mice model.

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ± SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	16	28		
Vehicle control	12	9.17 ± 1.83	23.60± 4.62			Nil	8.67±2.06
CHL- 16.0	12	2.00± 0.63	3.83± 0.98	40.00± 12.75	Nil	5	17.00±1.42
CHL - 16.0 + Cyproheptadine- 10.0	12	Nil	Nil	Nil	Nil	12	
CHL - 16.0 + Amitryptiline - 50.0	12	Nil	1.02± 0.60	14.00± 10.71	Nil	Nil	18.50±0.70
CHL- 16.0 + Verapamil - 50.0	12	0.07± 0.05	3.33± 1.03	19.67± 6.77	Nil	8	25.50±0.70
CHL - 16.0 + Amantidine 50.0	12	2.50± 0.55	5.67± 1.03	40.00± 14.58	Nil	8	18.00±4.24

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Table - 34 Resistance modulation studies against MEFLOQUINE RESISTANT isolate of P.yoelii nigeriensis (N-67)- Swiss mice model

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ±SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	16	28		
Vechile control	12	7.50±1.52	27.50±7.15			Nil	11.00±2.37
Mefloquine 8.0	12	2.17±0.75	8.33±2.58	32.00±13.95	Nil	4	16.50±2.08
MFQ - 8.0 + Cyproheptadine 10.0	12	Nil	Nil	0.50	Nil	12	
MFQ - 8.0 + Amitryptiline - 50.0	12	Nil	2.17±0.75	30.00±8.63	Nil	8	17.50±2.12
MFQ - 8.0 + Verapamil - 50.0	12	0.02±0.04	5.17±1.47	39.60±7.40	Nil	10	16.0±0.00
MFQ - 8.0 + Amantidine - 50.0	12	3.00±0.89	6.83±1.17	40.00±7.90	Nil	8	18.50±3.54

Animals inoculated with 1×10^7 parasites on Day 0

Observations upto day 28 post inoculation.

Table-35 Resistance modulation studies against HALOFANTRINE RESISTANT isolate of P.yoelii nigeriensis (N-67)

Swiss mice model

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ±SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	16	28		
Vehicle control	12	5.33±1.75	15.33±2.80	-		Nil	12.17±3.19
Halofantrine - 4.0	12	3.00±1.26	10.83±4.12	39.25±9.78	Nil	4	16.00±3.92
Halofantrine - 4.0 + Cyproheptadine - 10.0	12	Nil	Nil	Nil	Nil	12	
Halofantrine - 4.0 + Amitryptiline - 50.0	12	0.69±0.49	1.33±0.52	16.17±15.03	Nil	2	19.50±2.12
Halofantrine - 4.0 + Verapamil - 50.0	12	0.05±0.05	3.50±1.05	19.83±3.49	Nil	8	25.00±0.00
Halofantrine - 4.00 + Amantidine - 50.00	12	2.83±0.98	6.83±2.14	42.20±15.30	Nil	10	16.00±0.00

Animals inoculated with 1×10^7 parasites on day 0

Observations upto day 28 post inoculation

Table-36: Resistance modulation studies against PYRIMETHAMINE RESISTANT isolate of *P.yoelii nigeriensis* (N-67)

Swiss mice model

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ±SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	10	28		
Vehicle control	12	7.83±1.47	20.00±7.90	36.00±19.79		Nil	9.17±1.60
Pyrimethamine - 4.0	12	6.00±1.26	13.00±2.68	32.50±10.40		Nil	12.33±3.08
Pyrimethamine - 4.0 + Cyproheptadine - 10.0	12	2.83±0.98	11.67±5.32	35.00±11.18		Nil	12.00±2.00
Pyrimethamine - 4.0 + Amitryptline - 50.0	12	3.33±0.82	10.33±4.51	35.00±7.07		Nil	8.33±3.50

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Table-37 : P.Knowlesi-Rhesus Monkey Model

Modultion of mefloquine resistance by cyproheptadine

Treatment Regimen			Number of monkeys treated (Initial Parasitaemia	Response to treatment	
Mefloquine mg/kg x 3 days)	+	Cyproheptadine mg/kg x 5 days days)		Number* protected	Number Recrudesced (on day)
40.0	+	-	2 (03, 1.8%)	-	2(11, 16)
20.0	+	-	2 (0.3, 2.7%)	-	2 (9, 9)
20.0	+	10.0	2 (2.2, 2.8)	2	
20.0	+	5.0	3 (1.4, 1.6, 2.2%)	3	-
20.0	+	2.5	4 (1.3, 1.4,1.7,3.7%)	2	2 (13, 14)
20.0	+	1.25	6 (0.4,0.4,1.6,1.9 2.2,2.5%)	2	4 (9,11,11,16)
20.0	+	0.62	2 (2.3, 2.5%)	1	1 (9)
10.0	+	5.0	2 (0.8, 1.2)	2	-
10.0	+	2.5	2 (1.8, 2.6)	2	2 (4, 8)

* Monkeys which did not show recrudescence upto day 60 post treatment were recorded as 'Protected'

Table-38 : Comparison of uptake of different radioactive precursors in in vitro culture of P. cynomolgi and p.knowlesi using 6% haematocrit.

S.No.	Radiolabelled precursors	P.knowlesi(5-6%)	P.cynomolgi (2-3%)
1.	^{3}H Thymidine (0.5 µCi/well)	1348±278.50	1119.33±197.30
2.	^{3}H Leucine (1µCi/well)	12034±350.39	6190±272.42
3.	^{3}H Isoleucine (0.5µCi/well)	1979±181.5	4711.66±268.07
4.	^{3}H Hypoxanthine (0.5 µCi/well)	25769±3255.66	17404±733.33

Table-39 : Determination of optimum concentration of ^{3}H hypoxanthine during the *in vitro* growth of *P.knowlesi*.

	Percent parasitaemia	Radioactive uptake (DPM) 0.5 µCi	0.25 µCi	0.125 µCi
Expt.I	2.5%	16476.66±2675.25	9307-20±1047.60	6815.00±108.25
Expt.II	11%	23230±3140.0	9556.0±1248.80	-
	4%	25769.0±3255.0	14026.0±2076.0	-
	1%	17017.0±445.0	12321.0±992.0	-
	NRBC	606.00±84.85	343.00±32.52	-

Table 40: Incorporation of ^{3}H hypoxanthine during short term *in vitro* culture *P.knowlesi* ;

Effect of duration of incubation on uptake .

Culture condition		Hypoxanthine uptake counts (DPM) at	
Parasitaemia	haematocrit	4 hrs	24 hrs
EXPERIMENT-1			
9 %	6%	3806.66±628..0	14546.66±1375.80
	3%	2504.0±98.66	28562.6±1264.65
	115%	1228.6±86.93	34707.0±3559.75
	0.75%	796.33±96.77	37017.66±3698.97
3%	6%	1318.66±79.32	24236.0±2373.0
	3%	945.0±71.92	23573.0±1656.31
	1.5%	787.0±172.86	26473.3±1207.83
	0.75%	540.33±51.39	13263.3±1307.16
1.0%	6%	837.0±296.6	19223.6±2090.4
	3%	547.00±84.48	16954.0±542.87
	1.5%	565.66±104.40	8931.0±1541.16
	0.75%	507.50±61.51	3892.0±336.99
NRBC	6%	390.5±47.37	586.0±137.16
	3%	477.66±56.88	467.66±242.73
	1.5%	895.33±31.89	444.0±157.70
EXPERIMENT-II			
8%	6%	1602.33±319.64	17611.00±2509.40
	1.5%	818.00±2.82	28441.66±7884.83
3%	6%	621.33±102.88	15136.66±1486.89
	1.5%	373.66±24.84	4508.50±624.37
0.18%	6%	466.00±158.39	4600.00±1127.58
	1.5%	346.00±86.11	776.50±369.81
NRBC	6%	480.00±73.13	573.66±95.55
	1.5%	329.50±13.43	521.33±117.73

Table 41 Incorporation of ^{3}H-hypoxanthine during short term *in vitro* culture of *P. knowlesi*:

Effect of varible parasitaemia and haematocrit on the uptake.

Parasitaemia	Radioactive incorporation (DPM)			
	6%	3%	1.5%	0.75%
EXPERIMENT-I				
10%	14546.66±1375.8	28562.6±1264.65	34707.0±3559.75	37817.66±3698.
3%	24236.0±2373.0	23573.0±1656.31	26473.3±1207.83	13267.3±1307.1
1%	19223.6±2090.4	16954.0±542.87	8931.0±1541.16	3892.0±336.99
NRBC	586.0±137.16	467.66±242.73	444.0±137.70	437.66±195.19
EXPERIMENT-II				
8%	17611.00±2509.40		28441.66±3280.40	
3%	15136.66±1486.89		4508.50±624.37	
0.8%	4600.00±1127.58		776.50±3699.81	
NRBC	573.66±95.55		521.33±117.73	

Table 42 Evaluation of dose response of chloroquine during short term *in vitro* culture of *P.knowlesi.*

Chloroquine concentration (ug/ml)	Exp.1	Exp.2.	Exp.3.
10.00	622	587	1067
2.50	1354	707	988
.625	1041	527	896
.156	663	684	1063
.0390	1042	736	17823
.0097	1124	19877	22828
.0024	18086	28346	24209
.00060	21482	27084	26032
.00015	20426	26448	26031
Control	20581	30628	26996
IC 50	0.0236	0.036	0.0366
95% limit	(0.017–0.030)	(0.029–0.044)	(0.0283–0.0474)
IC 90	0.299	0.373	0.6371
95% limit	(0.211–0.421)	(0.272–0.513)	(0.4258–0.9532)

Table 43 In vitro methemoglobin estimation using mastomys erythrolysate as a source of hemoglobin (substrate).

S.No.	Additives	Molar concentrations	Hemolysate concentrations	Range of % of Met Hb
1.	Control(PBS)	Not applicable	20%	Nil
2.	$NaNO_2$	10 uM		5.1=8.6%
		100 uM	"	18-22.7%
		1000 uM		80-89%
		2.5 mM		100%
3.	Chloroquine	10^{-3} M		2.0-3.7%
		10^{-6} M		Nil
		10^{-9} M		Nil
4.	Primaquine	10 uM		3.0-4.5%
		100 uM		8.0-11.6%
		1000 uM (10^{-3} M)		23.8-29.6%
5.	4 mPQ	10^{-3} M		41.8-50%

Table- 44 :Prophylactic activity of rHU-IL-12 against challenge with sporozoites of P.cynomolgi B.

Group	Dose	No.of doses	Treatment days	Monkey no.	Result	Cure rate
1	100ng/kg	7	(-2 to+10)*	1	Patency on day 11	0/4
				2	Patency on day 11	
				3	Patency on day 12	
				4	Patency on day 11	
2.	1 ug/kg	7	(-2 to +10)*	1	Patency on day 13	0/4
				2	Patency on day 12	
				3	Patency on day 18	
				4	Patency on day 13	
3.	10 ug/kg	1	(-2)	1	Cured	4/4
				2	Cured	
				3	Cured	
				4	Cured	
4.	20 ug/kg	2	(-2 and 0)	1	Cured	2/2
				2	Cured	
5.	Nil	Nil		1.	Patency on day 10	0/4
				2	Patency on day 10	
				3	Patency on day 10	
				4	Patency on day 12	

*Treatment on alternate days

Table- 45 Prophylactic activity rHu-IL-12 (revalidation) against challenge with sporozoites of P.cynomolgi B.

Group	Dose	No.of doses	Treatment days	Monkey No.	Result	Cure rate
1	10ug/kg	1	(-2)	1	Cured	3/3
				2	Cured	
				3	Cured	
2.	Control	Nil			Patency on day 10	Nil

Table 46 Cytokine mRNA expression in controls, the groups that received multiple doses of rHuIL-12 (Grp 1 and 2), and the protected group that received a single dose (Grp 3) on the day of peak mRNA expression in Grp 3 for the 6 cytokines for which there was a significant elevation in monkeys that received rHuIL-12 as compared to controls.

(Assay carried out at CDC)

Cytokine	Day of Peak in Grp 3	Geometric Mean No. of Transcripts[a]			
		Contr Grp	Grp 1 100 ng/kg multiple doses	Grp 2 1 µg/kg multiple doses	Grp 3 10 µg/kg single dose
IL-6	2	1.8	1.0	2.4	26.0*
IL-10	2	7.7	23.2	31.4	82.2**
IL12α	0	1.0	1.0	2.1	11.4*
IL-15	0	1.0	1.0	1.0	8.5*
IFN-γ	4	2.3	24.5*	111.2[b]*	1809*
TNF-α	0***	13	12	59	71*

[a] Geometric mean transcripts on day of peak in the 10 µg/kg, single dose group (Grp3). Day of peak expression of mRNA was the same for all groups except for IFN-γ in Grp2 as noted below.

[b] Peak for this group was on day 2; the geometric mean number of transcripts on day 2 was 509.7.

* Significantly different ($p < 0.05$) from control (Mann-Whitney U test).

**p=0.059 for this group as compared to the control group by the Mann-Whitney U test. The p value for all other comparisons was > 0.10.

***Because TNF-α mRNA levels increased after sporozoite challenge in control and experimental groups (see text), we have only included data from monkeys prior to sporozoite challenge.

Table 47 Kinetics of cytokine mRNA expression in groups with significantly elevated levels: Fold Increase in mRNA expression over control monkey mRNA expression on same day.

		Day Relative to Sporozoite Challenge						
Cytokine	Group	Day -2	Day 0	Day 2	Day 4	Day 7	Day 11	Day 13
IL-6	3	1.0	1.0	14.6*	9.0	2.2	1.1	1.4
IL-10	3	1.0	4.0	10.6*	8.2	5.9	2.4	2.7
IL-12α	3	0.8	11.4*	2.6	0.6	1.2	1.8	1.2
IL-15	3	0.8	8.5*	1.9	1.6	1.0	1.0	1.0
IFNγ	1	0.8	6.4	24.2*	10.7*	1.1	1.8	2.2
	2	0.6	4.0	273.9*	48.8*	9.6	9.9	0.8
	3	0.8	23.0*	8.6*	792*	21.8*	7.7	3.9

* Number of transcipts in experimental group greater than in control monkeys ($p < 0.05$).

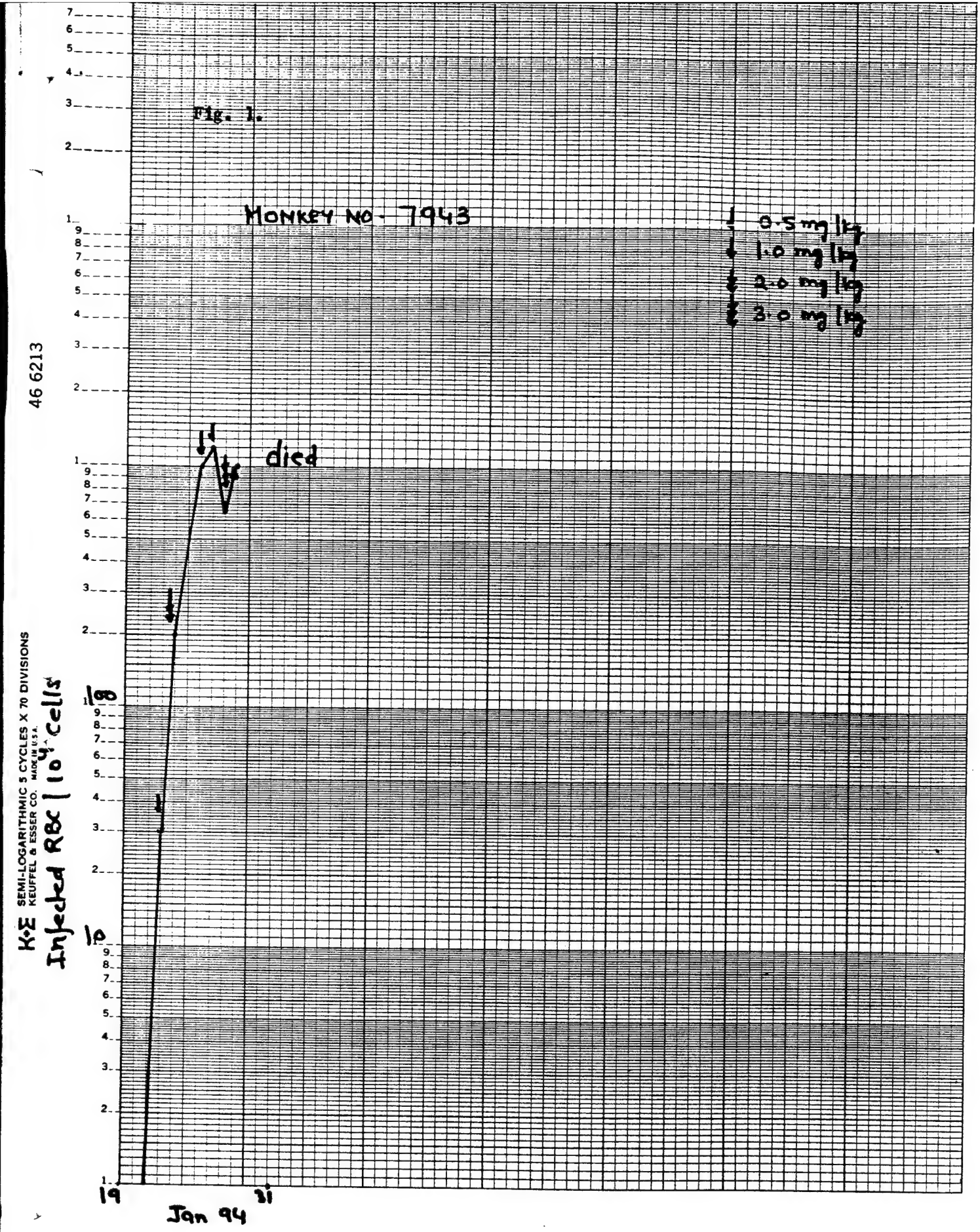


Fig. 1.
MONKEY NO. 7943
0.5 mg/kg
1.0 mg/kg
2.0 mg/kg
3.0 mg/kg
died
Infected RBC / 10^4 cells
100
10
19
31
Jan 94
46 6213
K+E SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

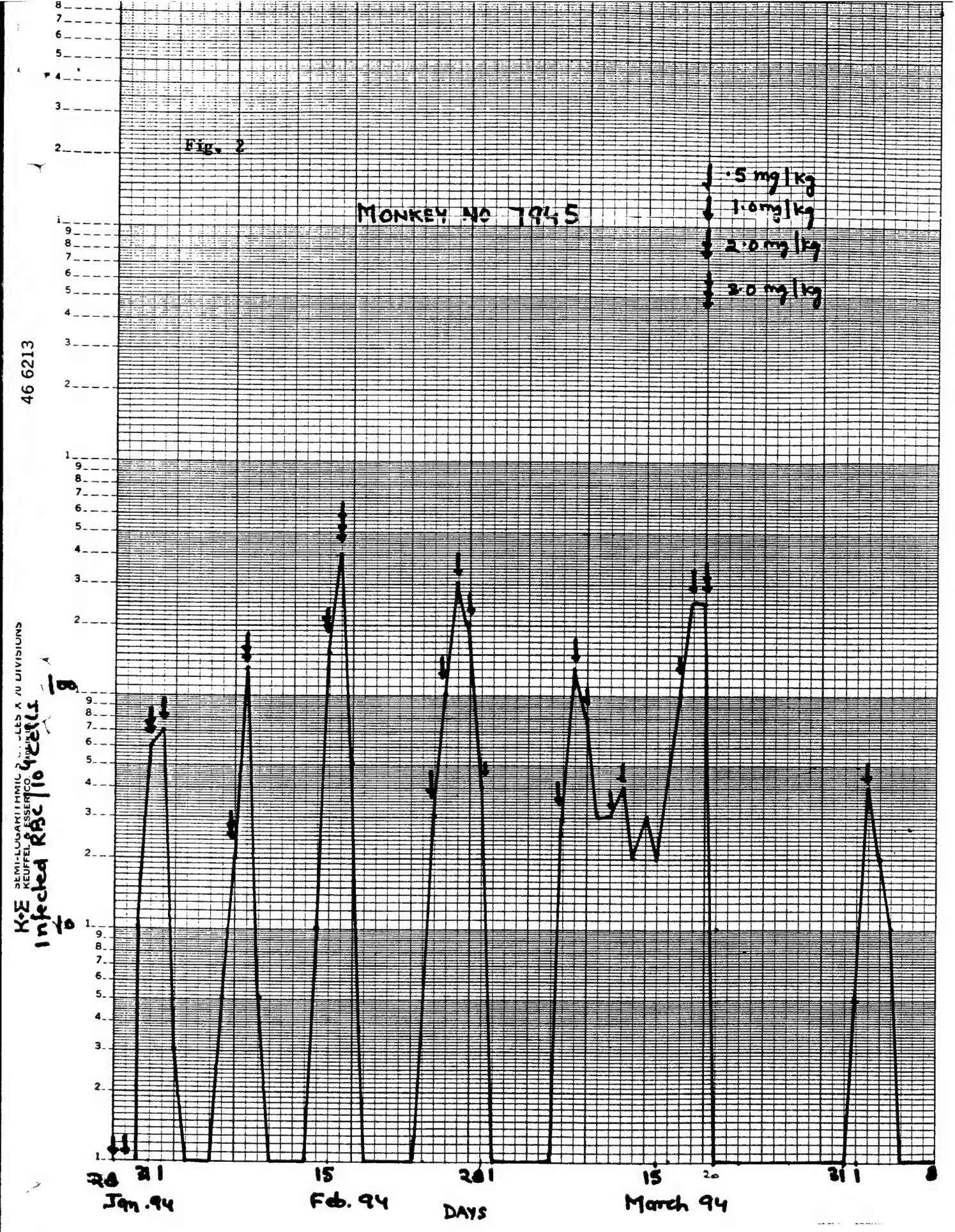


Fig. 2
MONKEY NO 7945
·5 mg/kg
1·0 mg/kg
2·0 mg/kg
3·0 mg/kg
K·E
Infected RBC/10 cells
DAYS
Jan. 94
Feb. 94
March 94
46 6213

Fig. 2 Contd..

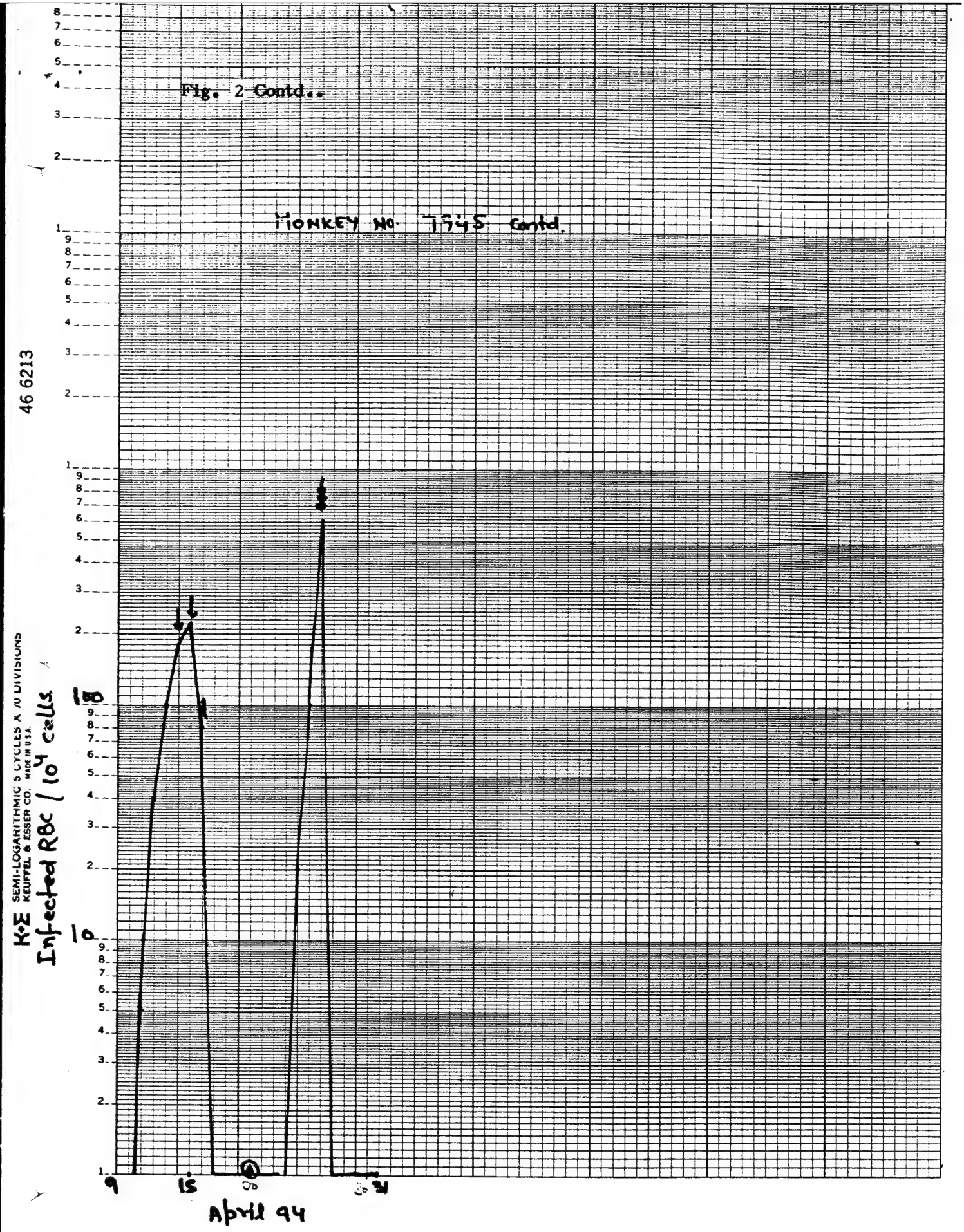
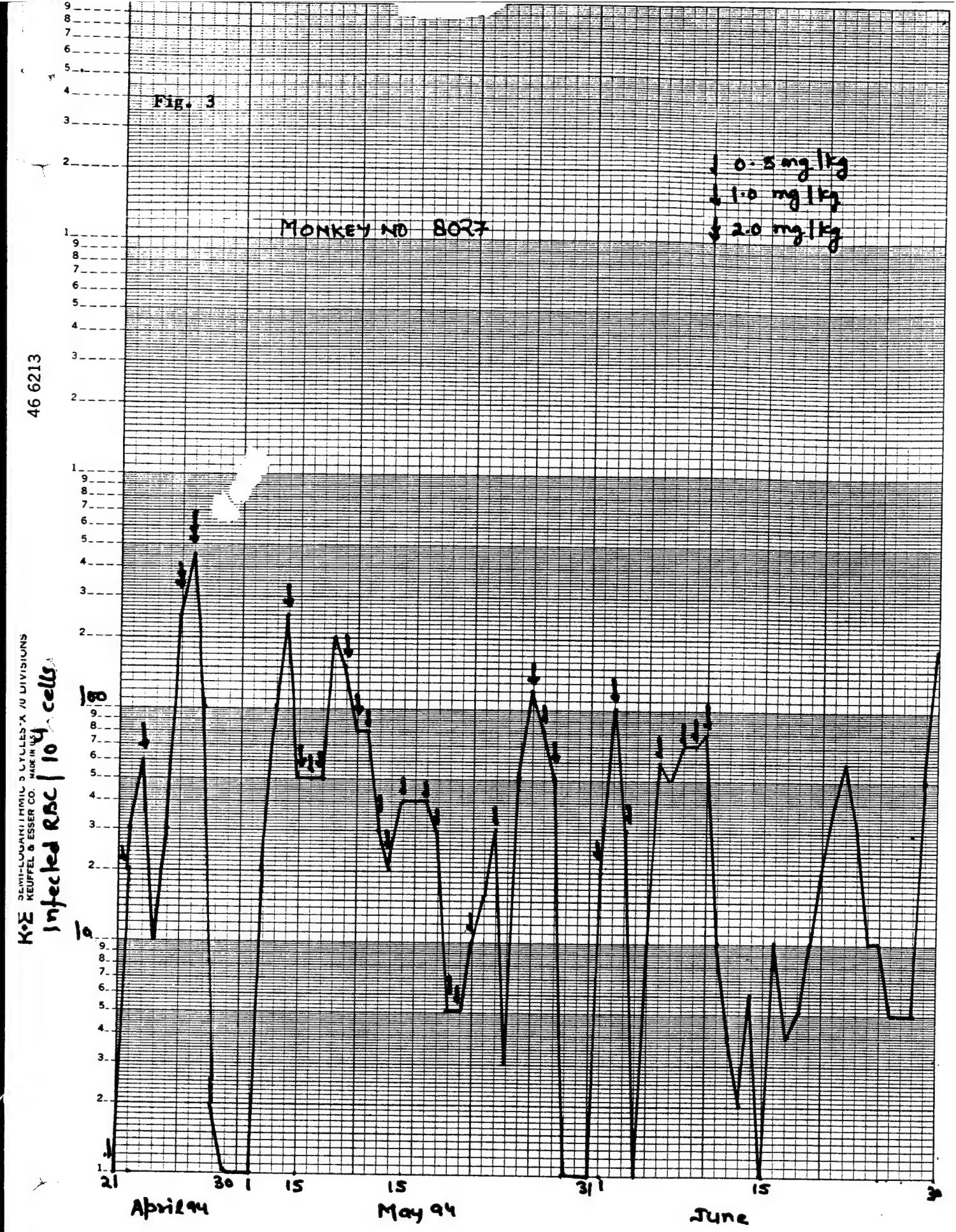


Fig. 3



46 6213
KE SEMI-LOGARITHMIC 3 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

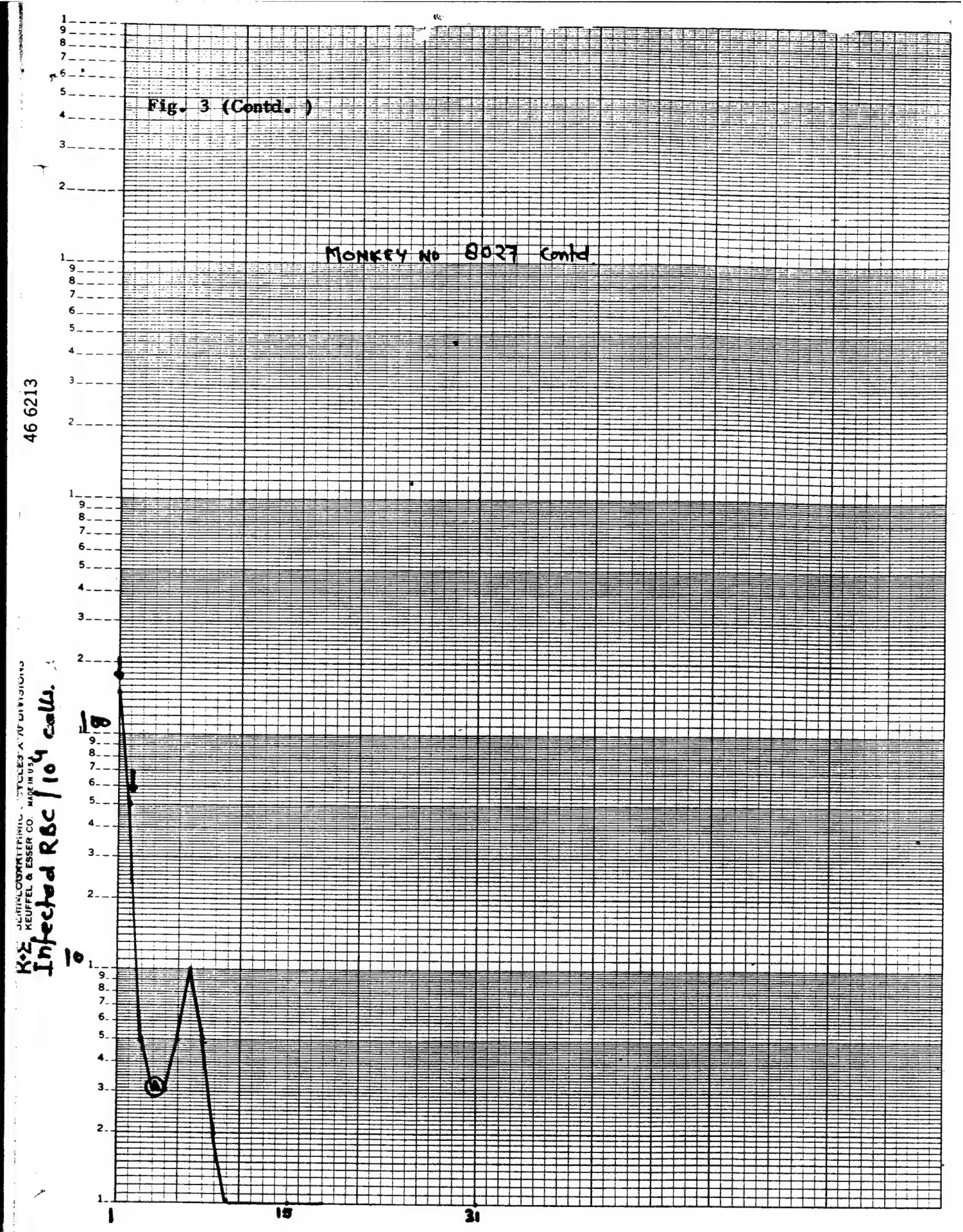
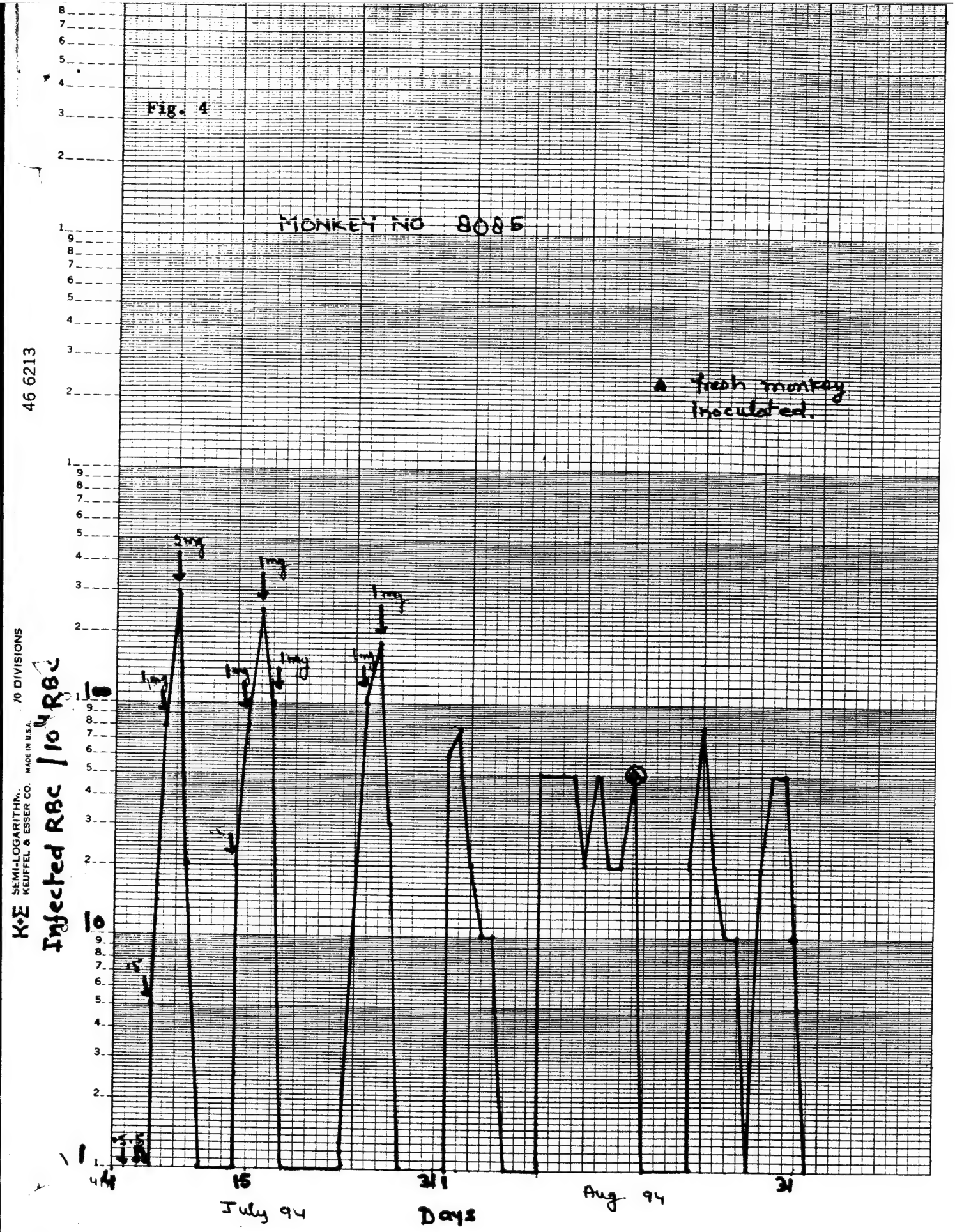


Fig. 3 (Contd.)
MONKEY NO 8027 Contd
Infected RBC / 10^4 cells.
10
1
15
31
46 6213
KEUFFEL & ESSER CO. MADE IN U.S.A.

Fig. 4


MONKEY NO 8085
▲ fresh monkey inoculated.
Injected RBC / 10^4 RBC
Days
July 94
Aug. 94
4
16
31
1
31
5mg
1mg
1mg
1

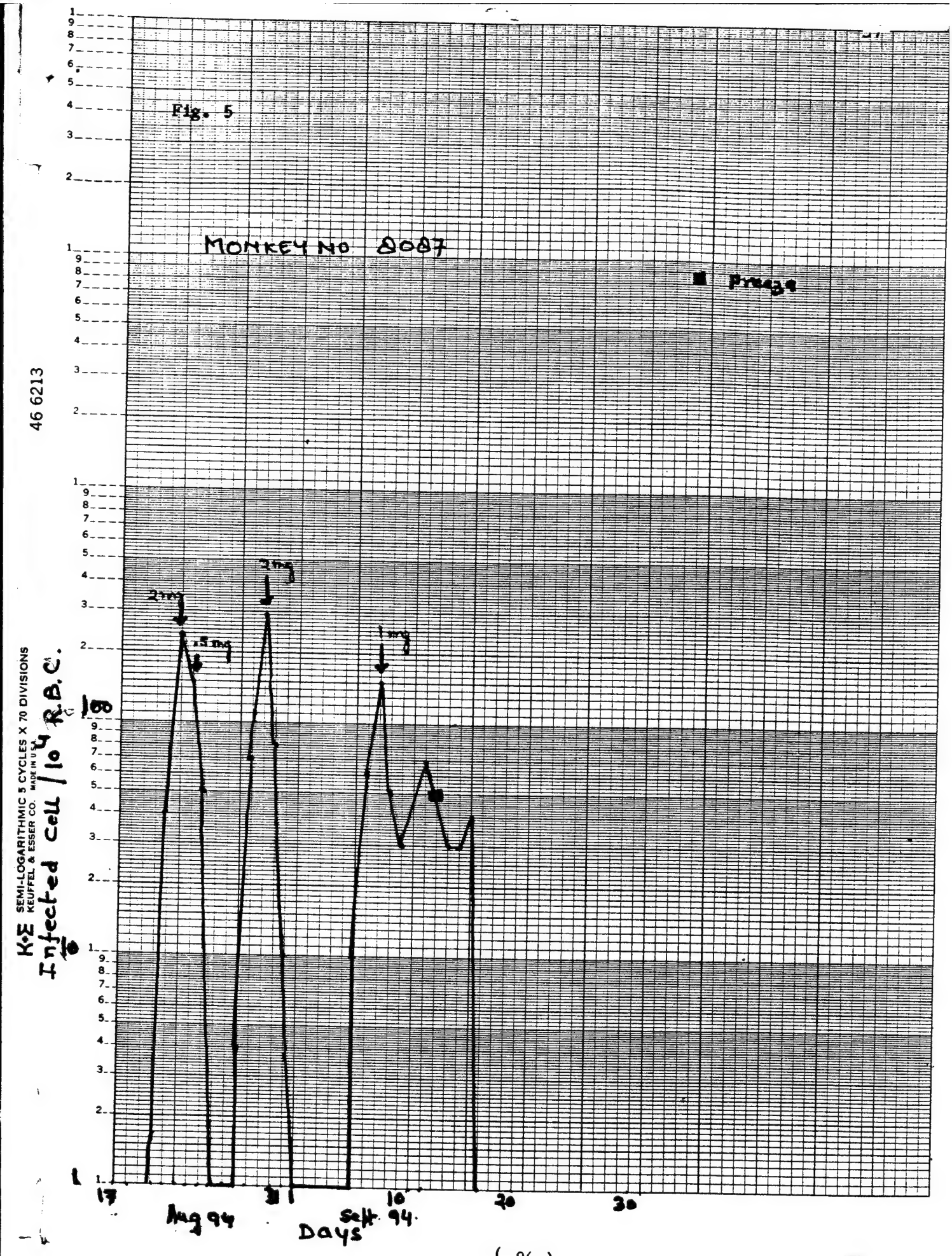


Fig. 5
MONKEY NO 8087
Freeze
2mg
.5mg
2mg
1mg
Infected Cell / 10^4 R.B.C.
100
10
1
17
31
10
20
30
Aug 94
Sept. 94
Days
K+E SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 6213

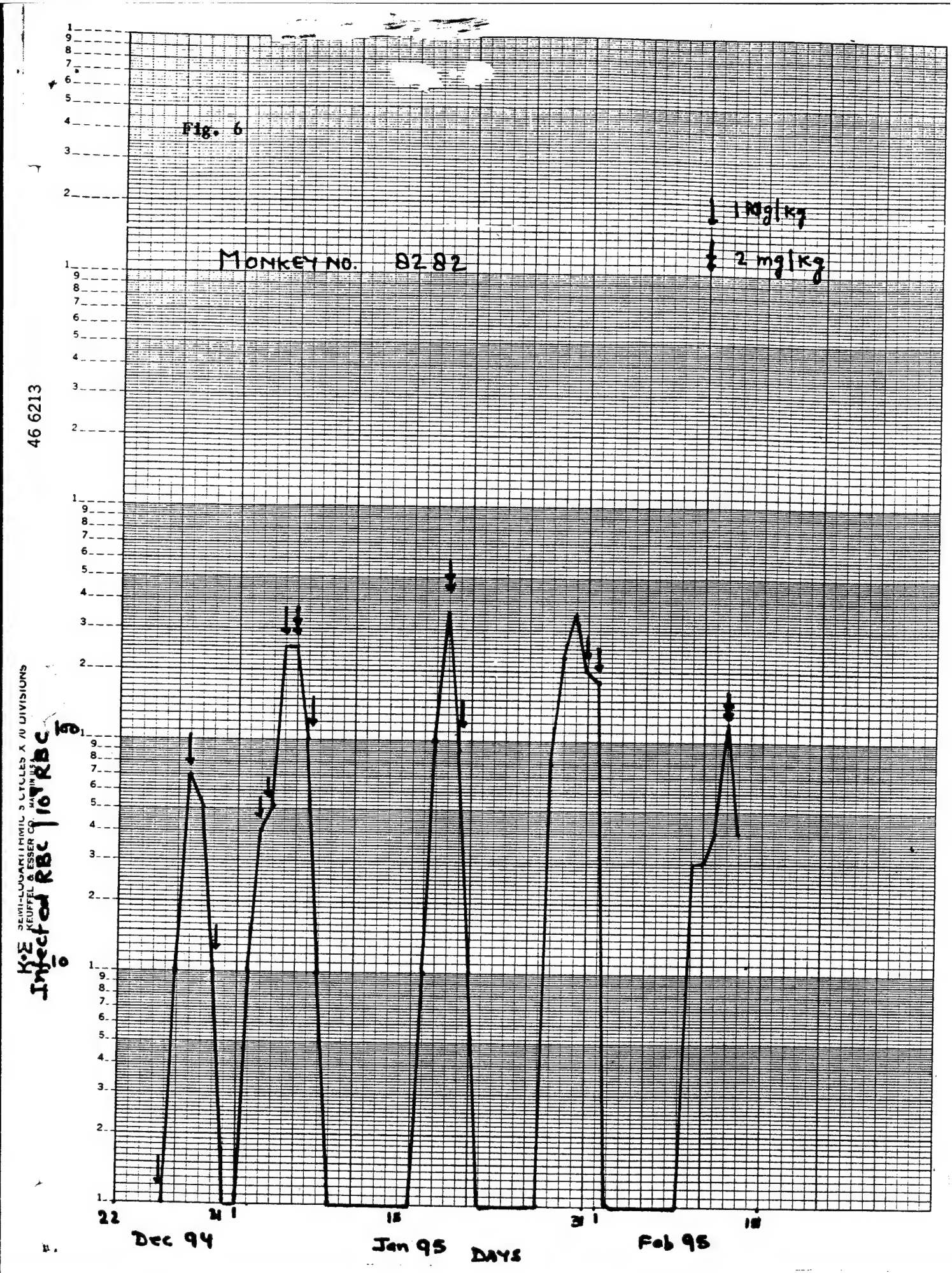
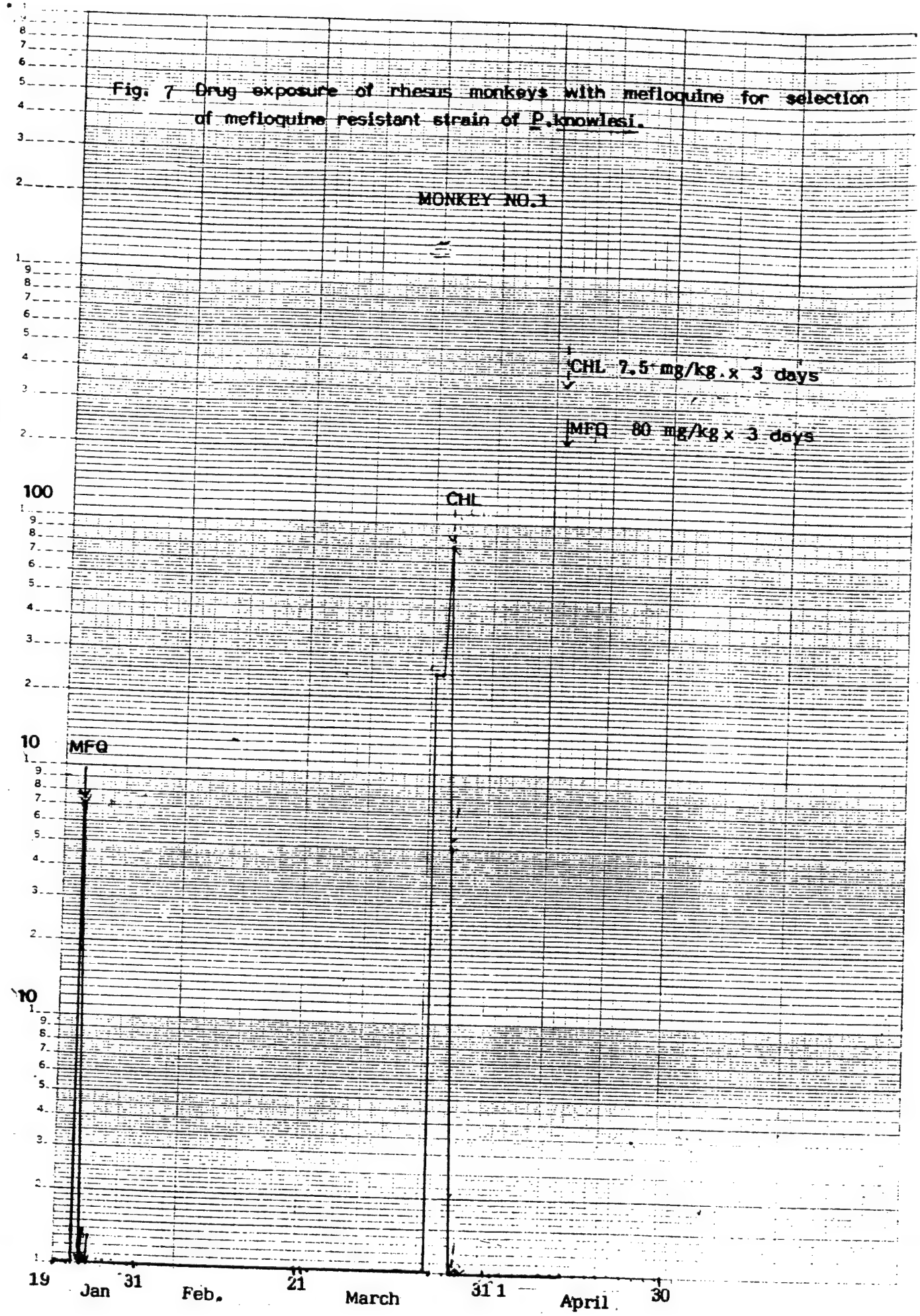


Fig. 6
MONKEY NO. 8282
1 mg/kg
2 mg/kg
46 6213
K·E
SEMI-LOGARITHMIC 3 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO.
Infected RBC / 10⁶ RBC
22
Dec 94
Jan 95
Feb 95
DAYS

Fig. 7 Drug exposure of rhesus monkeys with mefloquine for selection of mefloquine resistant strain of P.knowlesi.



SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

Fig. 8

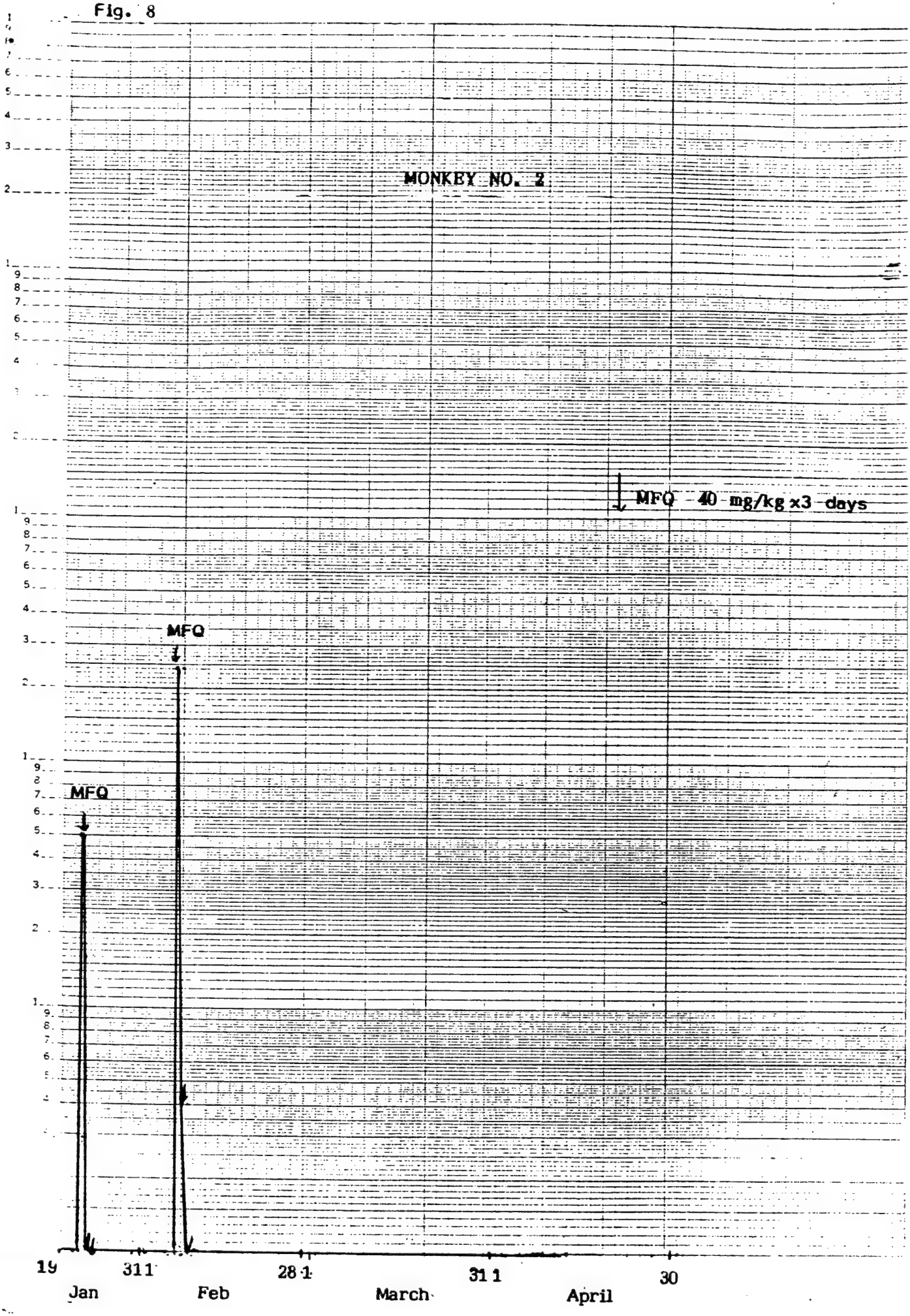
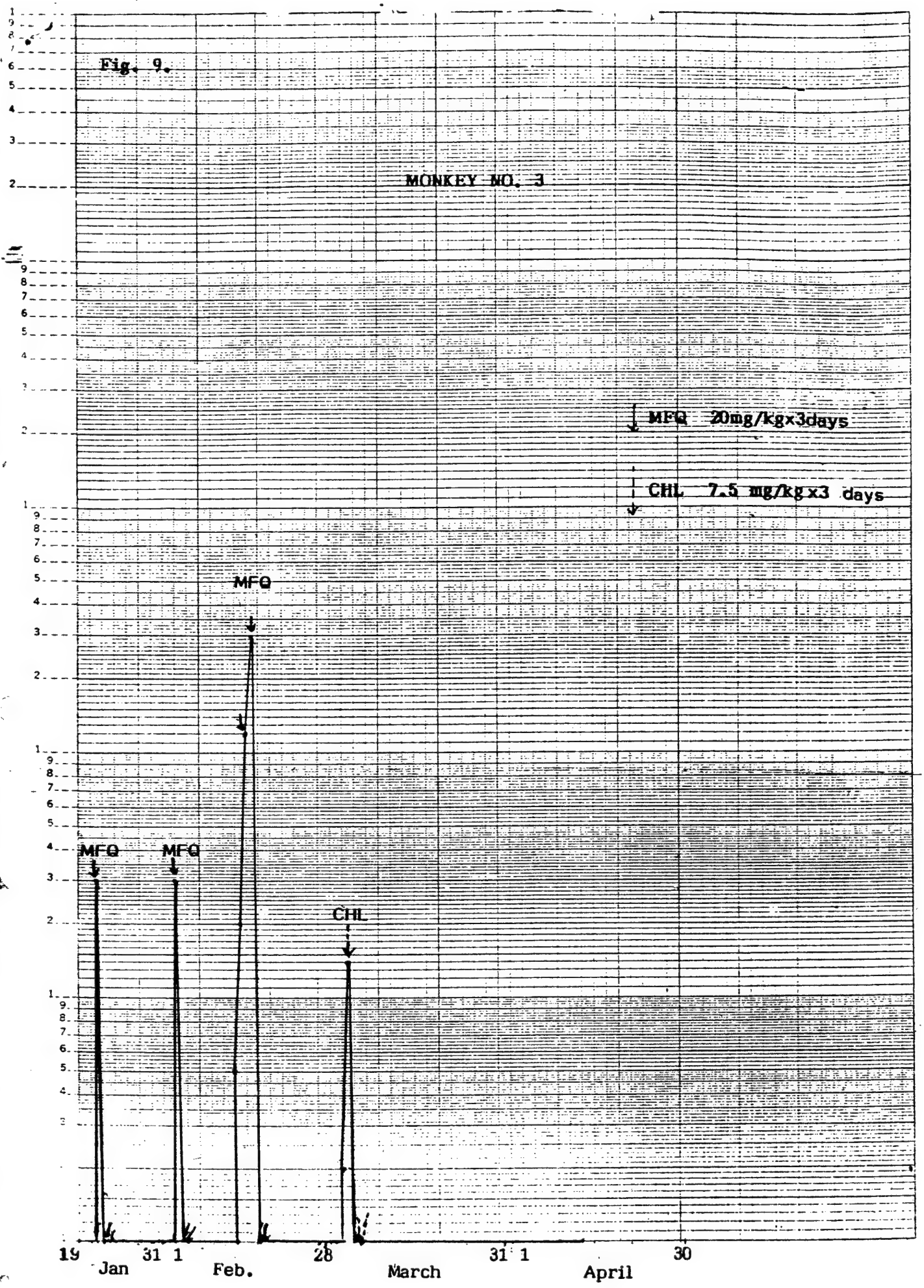

MONKEY NO. 2
MFQ 40 mg/kg x3 days
MFQ
MFQ
19
Jan
31 1
Feb
28 1
March
31 1
April
30

Fig. 9.



46 6213

KE SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

Fig. 10

P. knowlesi- Rhesus Monkey

Resistance Reversal Studies

Mefloquine+ Amitryptiline

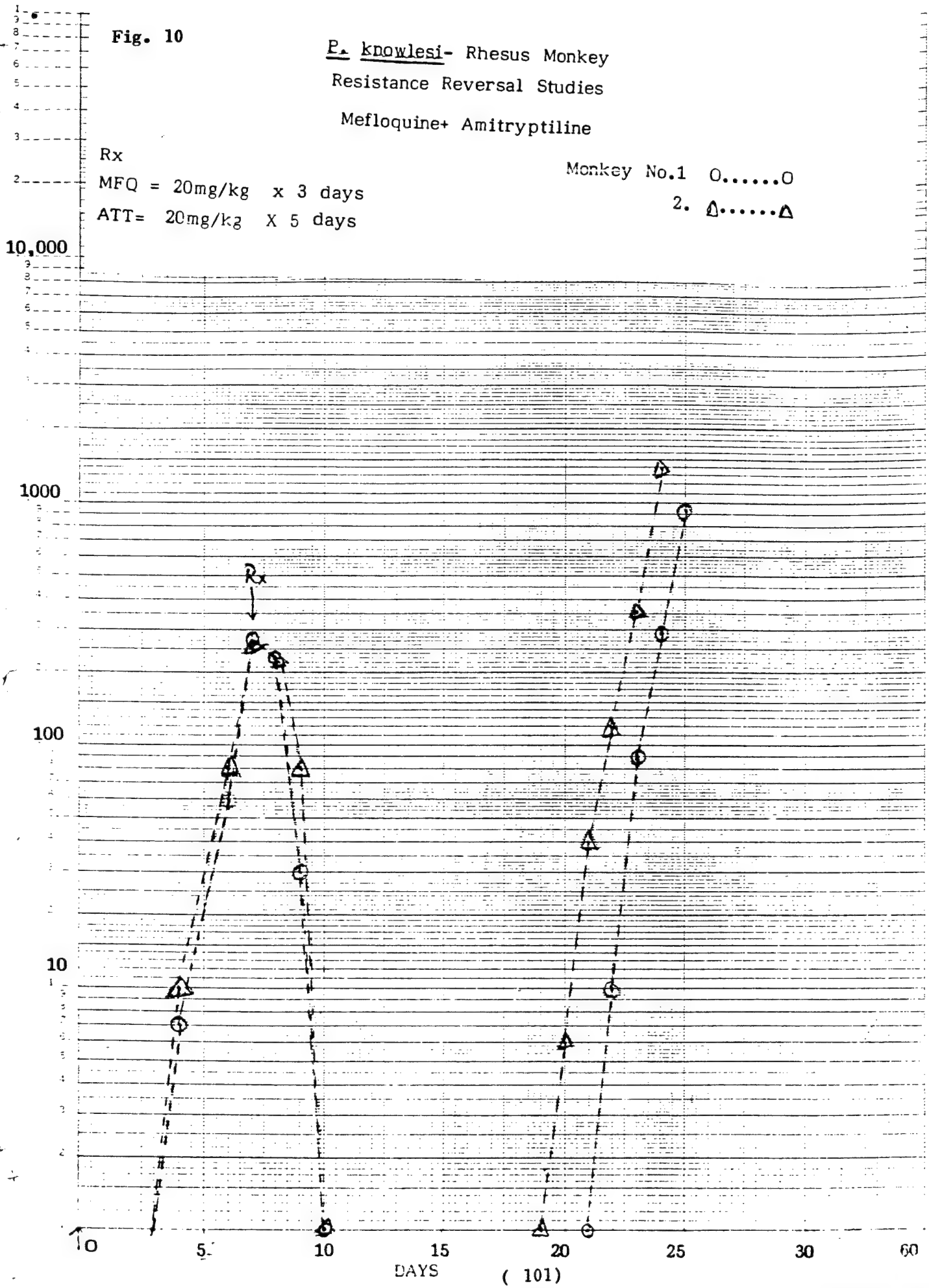
Rx

MFQ = 20mg/kg x 3 days

ATT= 20mg/kg X 5 days

Monkey No.1 O.....O

2. Δ.....Δ



46 6213

Fig. 11

P.knowlesi- Rhesus Monkey

Rx

Monkey No. 1. O.....O MFQ: 10mg/kgx3 days

2. △.....△ MFQ: 20mg/kgx3 days

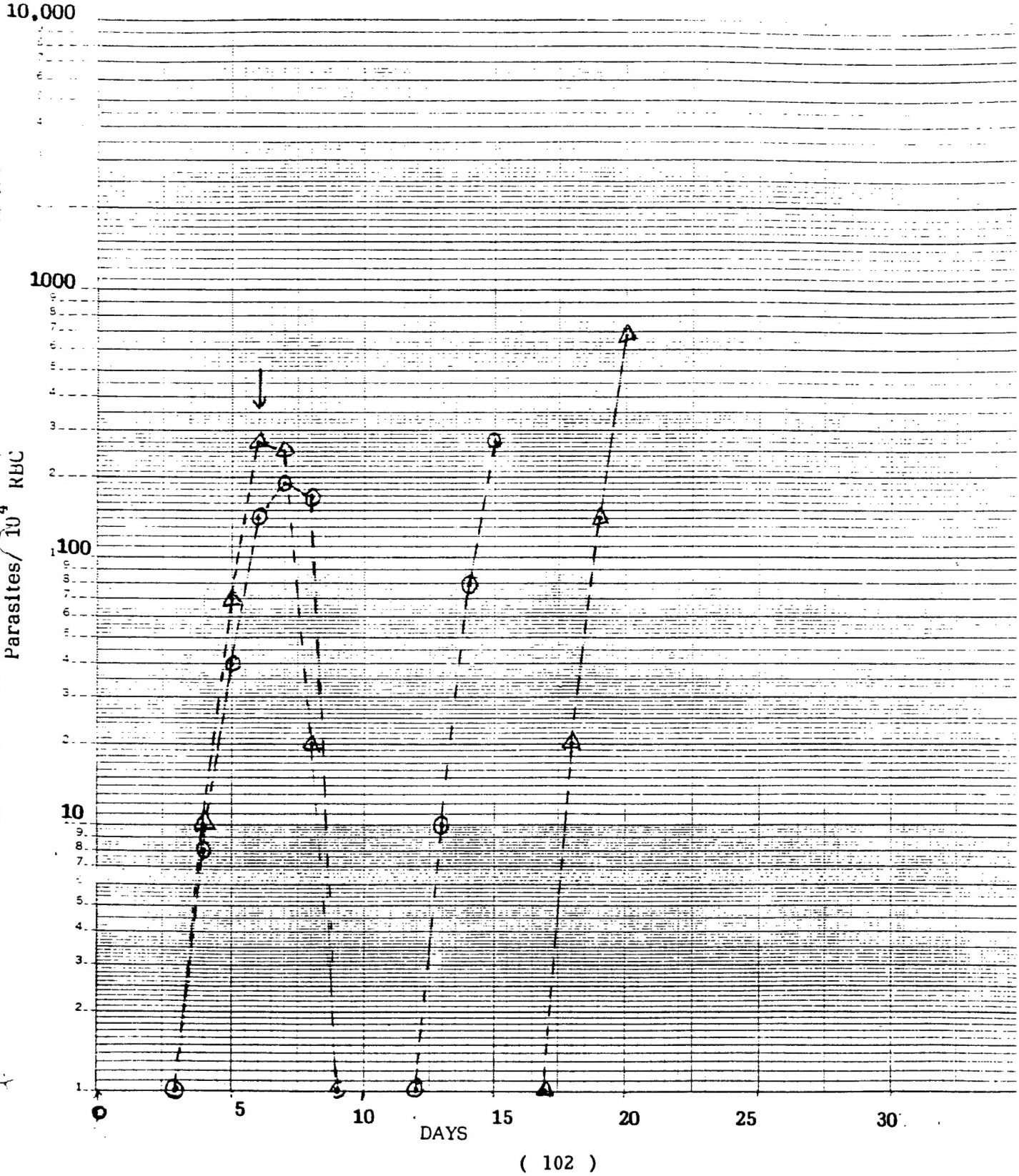
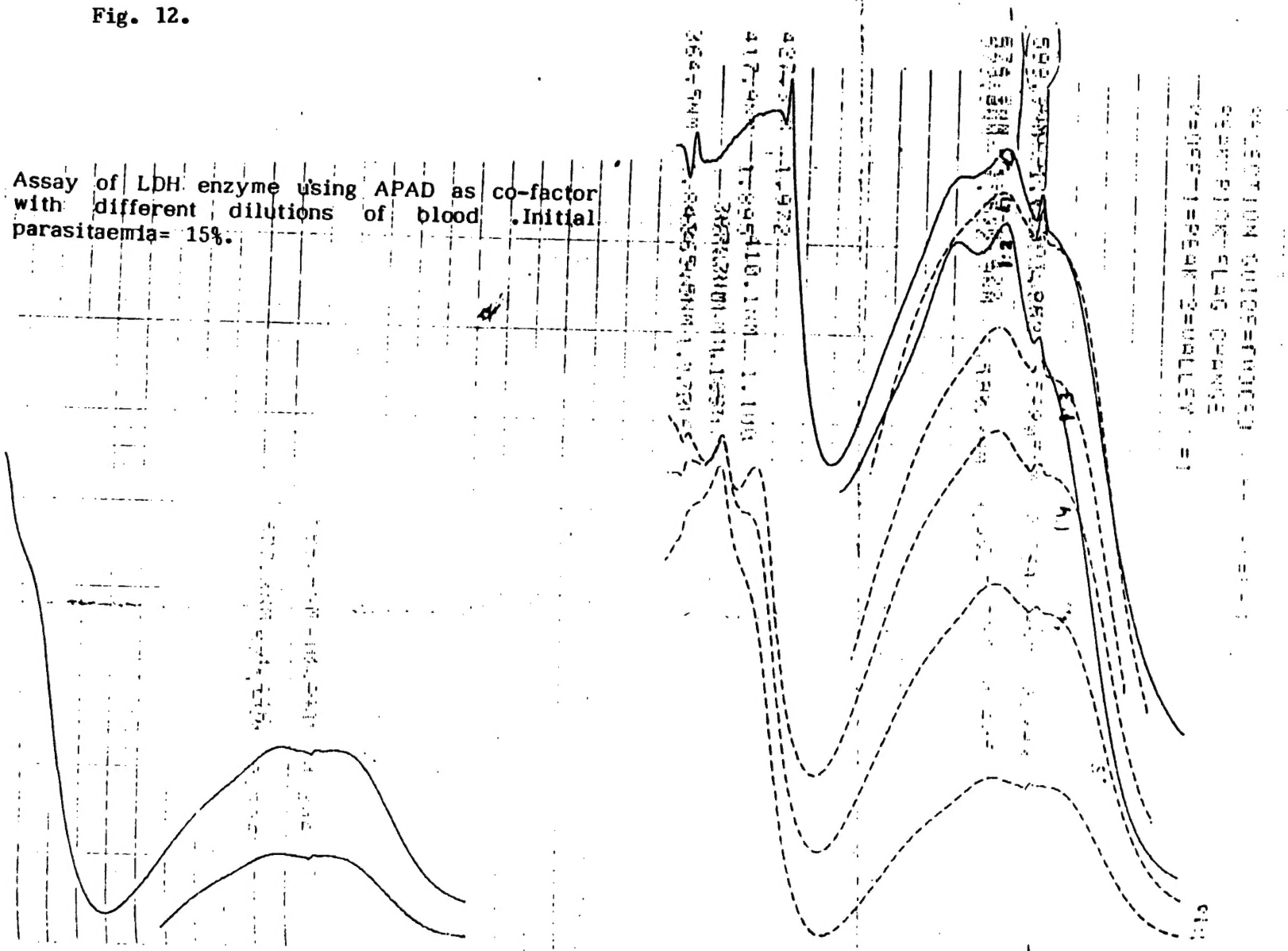


Fig. 12.

Assay of LDH enzyme using APAD as co-factor with different dilutions of blood .Initial parasitaemia= 15%.



Normal mouse blood

P. yoelii infected blood.

Fig. 13.

IL-12, exogenous, EIA
Control
Group 1, 0.1 µg/kg
Group 2, 1 µg/kg
Group 3, 10 µg/kg
Mean pg/ml (EIA) ± SEM
1000
100
10
1
-2
0
2
4
6
8
10
12
14
Days After Sporozoite Challenge

Fig. 14

IFN-γ, EIA
Control
Group 1, 0.1 µg/kg
Group 2, 1 µg/kg
Group 3, 10 µg/kg
Mean pg/ml (EIA) ± SEM
1000
100
10
1
-2
0
2
4
6
8
10
12
14
Days After Sporozoite Challenge